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# CONTENTS OF VOLUME 174

No. 1, MAY, 1948

	PAGE
GORANSON, E. S., HAMILTON, J. E., and HAIST, R. E. Changes in phosphate and carbohydrate metabolism in shock.....	1
SINGER, THOMAS P. On the mechanism of enzyme inhibition by sulfhydryl reagents.....	11
WITTNER, RUTH G., and PILLEMER, LOUIS. The immunochemistry of toxins and toxoids. V. The solubility of staphylococcal toxin in methanol-water mixtures under controlled conditions of pH, ionic strength, and temperature.....	23
BEILER, J. M., and MARTIN, GUSTAV J. Inhibition of hyaluronidase action by derivatives of hesperidin.....	31
KAPLAN, NATHAN O., and LIPMANN, FRITZ. The assay and distribution of coenzyme A.....	37
WINEGARD, HERBERT M., and TOENNIES, GERRIT. A method for computing the effective extraneous reducers in the Folin spectrophotometric determination of cystine.....	45
TITUS, ELWOOD, and FRIED, JOSEF. Counter-current distribution studies on streptomycin: the tautomerism of streptomycin.....	57
DANIEL, LOUISE J., SCOTT, M. L., HEUSER, G. F., and NORRIS, L. C. Further studies of unidentified factors required in the nutrition of <i>Lactobacillus casei</i> ..	71
MELCHIOR, JACKLYN B., MELLODY, MARGARET, and KLOTZ, IRVING M. The synthesis of protein by non-proliferating <i>Escherichia coli</i> .....	81
HEGSTED, D. MARK, and LIPMANN, FRITZ. The pantothenic acid content of coenzyme A by chick assay.....	89
GRANT, W. MORTON. Observations on blood acetol after administration of acetol, methyl alcohol, and formaldehyde to rabbits.....	93
ENGEL, FRANK L., PENTZ, E. IRENE, and ENGEL, MILDRED G. On the use of the nephrectomized rat for the study of rapid changes in nitrogen metabolism. . .	99
MULLINS, LORIN J., and ZERAHN, KARL. The distribution of potassium isotopes in biological material.....	107
OCHOA, SEVERO. Biosynthesis of tricarboxylic acids by carbon dioxide fixation. I. The preparation and properties of oxalosuccinic acid.....	115
OCHOA, SEVERO, and WEISZ-TABORI, ERNA. Biosynthesis of tricarboxylic acids by carbon dioxide fixation. II. Oxalosuccinic carboxylase.....	123
OCHOA, SEVERO. Biosynthesis of tricarboxylic acids by carbon dioxide fixation. III. Enzymatic mechanisms.....	133
KORNBERG, ARTHUR, OCHOA, SEVERO, and MEHLER, ALAN H. Spectrophotometric studies on the decarboxylation of $\beta$ -keto acids.....	159
MAGASANIK, BORIS, and CHARGAFF, ERWIN. The stereochemistry of an enzymatic reaction: the oxidation of <i>l</i> -, <i>d</i> -, and <i>epi</i> -inositol by <i>Acetobacter suboxydans</i> .....	173
SOMOGYI, MICHAEL. Studies of arteriovenous differences in blood sugar. I. Effect of alimentary hyperglycemia on the rate of extrahepatic glucose assimilation.....	189
ROE, JOSEPH H., MILLS, MARY B., OESTERLING, M. JANE, and DAMRON, CHARLOTTE M. The determination of diketo- <i>l</i> -gulonic acid, dehydro- <i>l</i> -ascorbic acid, and <i>l</i> -ascorbic acid in the same tissue extract by the 2,4-dinitrophenylhydrazine method.....	201

BARRY, GUY T., SATO, YOSHIO, and CRAIG, LYMAN C. Distribution studies. X. Attainment of equilibrium.....	209
SATO, YOSHIO, BARRY, GUY T., and CRAIG, LYMAN C. Distribution studies. XI. Isolation of benzylpenicillin containing radioactive sulfur.....	217
BARRY, GUY T., SATO, YOSHIO, and CRAIG, LYMAN C. Distribution studies. XII. Purity of crystalline penicillins.....	221
NOVAK, A. F., and HAUGE, S. M. Some properties of an unidentified growth factor in distillers' dried solubles.....	235
FERGER, MARTHA F., and DU VIGNEAUD, VINCENT. The microbial growth inhibition produced by optical isomers of $\beta$ -2-thienylalanine.....	241
NACHMANSOHN, DAVID, ROTHENBERG, MORTIMER A., and FELD, EMILY A. Studies on cholinesterase. V. Kinetics of the enzyme inhibition.....	247
BERNHEIM, FREDERICK, BERNHEIM, MARY L. C., and WILBUR, KARL M. The reaction between thiobarbituric acid and the oxidation products of certain lipides.....	257
JENSEN, ROBERT, SNELLMAN, OLLE, and SYLVÉN, BENGT. On the inhomogeneity of commercial heparin preparations from the physicochemical point of view..	265
KING, TSOO E., and CHELDELIN, VERNON H. Pantothenic acid studies. IV. Propionic acid and $\beta$ -alanine utilization.....	273
COHEN, SEYMOUR S. The synthesis of bacterial viruses. I. The synthesis of nucleic acid and protein in <i>Escherichia coli</i> B infected with $T_2r^+$ bacteriophage..	281
COHEN, SEYMOUR S. The synthesis of bacterial viruses. II. The origin of the phosphorus found in the desoxyribonucleic acids of the $T_2$ and $T_4$ bacteriophages.....	295
HOLZMAN, GEORGE, and NIEMANN, CARL. A spectrophotometric study of blood group A specific substance isolated from hog gastric mucosa.....	305
OLSON, O. E., FAGER, E. E. C., BURRIS, R. H., and ELVEHJEM, C. A. Folic acid activity in homogenates of rat liver.....	319
WOOLLEY, D. W., and PRINGLE, A. Some structural analogues antagonistic to pteroylglutamic acid.....	327
LITTLE, JOHN E., SPROSTON, THOMAS J., and FOOTE, MURRAY W. Isolation and antifungal action of naturally occurring 2-methoxy-1,4-naphthoquinone...	335
SINCLAIR, R. G. The lecithin, cephalin, and sphingomyelin content of serum. I. As indicated by the choline-phosphorus and nitrogen-phosphorus ratios...	343
SINCLAIR, R. G. The lecithin, cephalin, and sphingomyelin content of serum. II. As indicated by the amino nitrogen and non-diffusible phosphorus content after differential hydrolysis of the acetone-insoluble lipides.....	355
DOUNCE, ALEXANDER L., ROTHSTEIN, ASER, BEYER, G. THANNHAUSER, MEIER, REBECCA, and FREER, RICHARD M. A detailed procedure for the preparation of highly purified adenosine triphosphate.....	361
HOROWITZ, N. H., and SRB, ADRIAN M. Growth inhibition of <i>Neurospora</i> by canavanine, and its reversal.....	371

#### Letters to the Editors

ROBERTS, SIDNEY, ADAMS, ELIJAH, and WHITE, ABRAHAM. Release of antibody by lymphoid tissue <i>in vitro</i> .....	379
MAW, GEORGE A., and DU VIGNEAUD, VINCENT. Dimethyl- $\beta$ -propiothetin, a new methyl donor.....	381
SCHWEIGERT, B. S., GERMAN, H. L., and GARBER, M. J. Synthesis of nicotinic acid from tryptophan by the developing chick embryo.....	383

BINKLEY, FRANCIS. Birefringence of flow of preparations of myosin.....	385
KIELLEY, W. WAYNE, and MEYERHOF, OTTO. A new magnesium-activated adenosinetriphosphatase from muscle.....	387
COHEN, PHILIP P., and GRISOLIA, SANTIAGO. The intermediate rôle of carhamyl-1-glutamic acid in citrulline synthesis.....	389

## No. 2, JUNE, 1948

KOBAYASHI, YUTAKA, FLING, MARGUERITE, and FOX, SIDNEY W. Antipodal specificity in the inhibition of growth of <i>Escherichia coli</i> by amino acids...	391
SALTZMAN, ABRAHAM. Fluorophotometric method for the estimation of salicylate in blood.....	399
COLIO, LLOYD G., and BABB, VIRGINIA. Study of a new stimulatory growth factor.....	405
SCHWERT, GEORGE W. A note on the application of the colorimetric ninhydrin determination to studies of enzyme kinetics.....	411
CARTER, HERBERT E., BELINSKEY, CAROL, CLARK, R. K., JR., FLYNN, EDWIN H., LYTTLE, BETTY, McCASLAND, G. E., and ROBBINS, MARY. Oxidation of inositol by <i>Acetobacter suboxydans</i> .....	415
REID, JAMES C., and JONES, HARDIN B. Radioactivity distribution in the tissues of mice bearing melanosarcoma after administration of DL-tyrosine labeled with radioactive carbon.....	427
FOLCH, JORDI. The chemical structure of phosphatidyl serine.....	439
TOENNIES, GERRIT, and GALLANT, DOROTHY LEAF. Bacterimetric studies. I. Factors affecting the precision of bacterial growth responses and their measurement.....	451
BODANSKY, OSCAR. The inhibitory effects of DL-alanine, L-glutamic acid, L-lysine, and L-histidine on the activity of intestinal, bone, and kidney phosphatases.....	465
DE VIGNEAUD, VINCENT, MOYER, A. W., and CHANDLER, JOSEPH P. Dimethyl-thetin as a biological methyl donor.....	477
LAUFFER, MAX A. Interaction between tobacco mosaic virus and bovine serum albumin.....	481
SHAFFER, C. BOYD, and CRITCHFIELD, FRANCES H. Lipotropic activity and toxicity of methoxinine (oxymethionine).....	489
MARTIN, GUSTAV J., AVAKIAN, SOUREN, and MOSS, JACK. Studies of pyridoxine displacement.....	495
NAGANNA, B., and NARAYANA MENON, V. K. Erythrocyte pyrophosphatase in health and disease. I. Properties of the enzyme.....	501
FRIEDGOOD, HARRY B., GARST, JOSEPHINE B., and HAAGEN-SMIT, A. J. A new method for the separation of androgens from estrogens and for the partition of estriol from the estrone-estradiol fraction. With special reference to the identification and quantitative microdetermination of estrogens by ultraviolet absorption spectrophotometry.....	523
WOLFF, J., and CHAIKOFF, I. L. Plasma inorganic iodide as a homeostatic regulator of thyroid function.....	555
WARE, ARNOLD G., and SEEGER, WALTER H. Studies on prothrombin: purification, inactivation with thrombin, and activation with thromboplastin and calcium.....	565
IRVIN, J. LOGAN, and IRVIN, ELINOR MOORE. Apparent dissociation exponents of quinine, pamaquine, and a quinolylpiperidylcarbinol; application of an extended pH scale.....	577

IRVIN, J. LOGAN, and IRVIN, ELINOR MOORE. A fluorometric method for the determination of pamaquine, SN-13276, and SN-3294.....	589
SOMOGYI, MICHAEL. Studies of arteriovenous differences in blood sugar. II. Effect of hypoglycemia on the rate of extrahepatic glucose assimilation.....	597
JUNQUEIRA, PLINIO B., and SCHWEIGERT, B. S. Further studies on the rôle of vitamin B <sub>6</sub> in the metabolism of tryptophan by the rat.....	605
BIRD, H. R., RUBIN, MAX, and GROSCHKE, A. C. A chick growth factor in cow manure. VII. Its stability and solubility.....	611
DORFMAN, ALBERT, OTT, MELVIN L., and WHITNEY, RICHARD. The hyaluronidase inhibitor of human blood.....	621
WILLIAMSON, MARTIN B. The vitamin A content of fetal rats from mothers on a high cholesterol diet.....	631
BUBL, EDWARD C., and BUTTS, JOSEPH S. The utilization of D-tyrosine for growth in the rat.....	637
LING, KUO-HUANG, and TUNG, TA-CHENG. The oxidative demethylation of monomethyl-L-amino acids.....	643
NOVAK, A. F., and HAUGE, S. M. Isolation of the unidentified growth factor (vitamin B <sub>12</sub> ) in distillers' dried solubles.....	647
TERESI, J. D., and LUCK, J. MURRAY. The combination of organic anions with serum albumin. VI. Quantitative studies by equilibrium dialysis.....	653
BISCHOFF, FRITZ, and PILHORN, HARRY R. The state and distribution of steroid hormones in biologic systems. III. Solubilities of testosterone, progesterone, and $\alpha$ -estradiol in aqueous salt and protein solution and in serum.....	663
GJESSING, ERLAND C., LUDEWIG, STEPHAN, and CHANUTIN, ALFRED. Fractionation, electrophoresis, and chemical studies of proteins in sera of control and injured goats.....	683
SAUNDERS, P. R., SIU, R. G. H., and GENEST, R. N. A cellulolytic enzyme preparation from <i>Myrothecium verrucaria</i> .....	697
SIMMONDS, SOFIA, and FRUTON, JOSEPH S. The utilization of proline derivatives by mutant strains of <i>Escherichia coli</i> .....	705
SIMMONDS, SOFIA. Utilization of sulfur-containing amino acids by mutant strains of <i>Escherichia coli</i> .....	717
CARTER, HERBERT E., and LOO, Y. H. Oxidation of hydroxy-L-proline by periodate.....	723
LORING, HUBERT S., ROLL, PAUL M., and PIERCE, JOHN G. A simplified procedure for the preparation of cytidylic acid and diammonium uridylate from yeast nucleic acid.....	729
RAPPORT, MAURICE M., GREEN, ARDA ALDEN, and PAGE, IRVINE H. Partial purification of the vasoconstrictor in beef serum.....	735
HARDING, W. M., and SHIVE, WILLIAM. Biochemical transformations as determined by competitive analogue-metabolite growth inhibitions. VIII. An interrelationship of methionine and leucine.....	743

#### *Letters to the Editors*

FRIEDKIN, MORRIS, and LEHNINGER, ALBERT L. Phosphorylation coupled to electron transport between dihydrodiphosphopyridine nucleotide and oxygen....	757
OVERMAN, RALPH S., and WRIGHT, IRVING S. A new blood clotting inhibitor..	759
KUN, ERNEST. Inhibition of the phosphorylation of glucose by meningococcal endotoxin.....	761
SEALOCK, ROBERT RIDGELY, and LEPOW, JEAN PAPELL. Antipernicious anemia extracts and tyrosine metabolism in the scorbutic guinea pig.....	763

HITCHINGS, GEORGE H., ELION, GERTRUDE B., VANDERWERFF, HENRY, and FALCO, ELVIRA A. Pyrimidine derivatives as antagonists of pteroylglutamic acid. ....	765
GRINSTEIN, MOISÉS, KAMEN, MARTIN D., and MOORE, CARL V. Observation on the utilization of glycine in the biosynthesis of hemoglobin. ....	767
WILLIAMSON, MARTIN B., and APPEL, MURIEL. The effect of sulfonamide in the diet of pregnant rats on erythropoiesis in the fetuses. ....	769
KAICKAR, HERMAN M., KJELDGAARD, NIELS OLE, and KLENOW, HANS. Inhibition of xanthine oxidase and related enzymes by 6-pteridyl aldehyde. ....	771
FRANTZ, IVAN D., JR., ZAMECNIK, PAUL C., REESE, J. W., and STEPHENSON, MARY L. The effect of dinitrophenol on the incorporation of alanine labeled with radioactive carbon into the proteins of slices of normal and malignant rat liver. ....	773

## No. 3, JULY, 1948

PETERSON, MERLIN H., and JOHNSON, MARVIN J. The estimation of fatty acids of intermediate chain length by partition chromatography. ....	775
BURCH, HELEN B., LOWRY, OLIVER H., BESSEY, OTTO A., and BERSON, BELLA Z. The determination of iron in small volumes of blood serum. ....	791
LING, CHIUN-TONG, HEGSTED, D. MARK, and STARE, F. J. The effect of pyridoxine deficiency on the tryptophan-niacin transformation in rats. ....	803
McGILVERY, R. W., and COHEN, PHILIP P. The decarboxylation of L-phenylalanine by <i>Streptococcus faecalis</i> R. ....	813
HALPERN, GEORGE R., and BIELY, JACOB. The utilization of vitamin A in various carriers. ....	817
FRAENKEL-CONRAT, HEINZ, and OLCOTT, HAROLD S. Reaction of formaldehyde with proteins. VI. Cross-linking of amino groups with phenol, imidazole, or indole groups. ....	827
KIBRICK, ANDRE C. A polariscopic study of glutamic acid and pyrrolidonecarboxylic acid. ....	845
GUTMANN, HELMUT R., and FRUTON, JOSEPH S. On the proteolytic enzymes of animal tissues. VIII. An intracellular enzyme related to chymotrypsin. ....	851
DOUNCE, ALEXANDER L., and BEYER, G. THANNHAUSER. The arginase activity of isolated cell nuclei. ....	859
HANKES, L. V., HENDERSON, L. M., BRICKSON, W. L., and ELVEHJEM, C. A. Effect of amino acids on the growth of rats on niacin-tryptophan-deficient rations. ....	873
MITCHELL, HERSCHEL K., and HOULAHAN, MARY B. An intermediate in the biosynthesis of lysine in <i>Neurospora</i> . ....	883
ARMSTRONG, MARVIN D., and BINKLEY, FRANCIS. On the failure of DL-homoserine to support growth on a methionine-deficient diet. ....	889
VOLCANI, BENJAMIN E., and SNELL, EDMOND E. The effects of canavanine, arginine, and related compounds on the growth of bacteria. ....	893
LESTER, DAVID, and GREENBERG, LEON A. The determination of acetone and ketone bodies. ....	903
COMAR, C. L., DAVIS, GEORGE K., and SINGER, LEON. The fate of radioactive copper administered to the bovine. ....	905
PERLMUTTER, MARTIN, and GREEP, ROY O. The uptake of glucose and the synthesis of glycogen by the isolated diaphragm of normal and pituitectomized rats. ....	915
HAINES, WILLIAM J., JOHNSON, RICHARD H., GOODWIN, MARI P., and KUIZENGA,	



MARVIN H. Biochemical studies on hog testicular extract. I. Isolation and identification of 5-pregnen-3( $\beta$ )-ol-20-one. Plate 1.....	925
KRAHL, M. E., and PARK, C. R. The uptake of glucose by the isolated diaphragm of normal and hypophysectomized rats.....	939
WHITELEY, ARTHUR H. A microvolumetric Van Slyke blood gas apparatus....	947
MEHLER, ALAN H., KORNBERG, ARTHUR, GRISOLIA, SANTIAGO, and OCHOA, SEVERO. The enzymatic mechanism of oxidation-reductions between malate or isocitrate and pyruvate.....	961
OCHOA, SEVERO, MEHLER, ALAN H., and KORNBERG, ARTHUR. Biosynthesis of dicarboxylic acids by carbon dioxide fixation. I. Isolation and properties of an enzyme from pigeon liver catalyzing the reversible oxidative decarboxylation of <i>l</i> -malic acid.....	979
HUEBNER, CHARLES F., and JACOBS, WALTER A. The aconite alkaloids. XXI. Further oxidation studies with atisine and isoatisine.....	1001
BOYD, M. JOHN, LOGAN, MILAN A., and TYTELL, ALFRED A. The growth requirements of <i>Clostridium perfringens (welchii)</i> BP6K.....	1013
BOYD, M. JOHN, LOGAN, MILAN A., and TYTELL, ALFRED A. A microbiological procedure for the assay of amino acids with <i>Clostridium perfringens (welchii)</i> BP6K.....	1027
<i>Letters to the Editors</i>	
HITCHINGS, GEORGE H., ELION, GERTRUDE B., and VANDERWERFF, HENRY. The limitations of inhibition analysis.....	1037
STADTMAN, E. R., and BARKER, H. A. Two new reactions producing acetyl phosphate.....	1039
BOROOK, HENRY, DEASY, CLARA L., HAAGEN-SMIT, A. J., KEIGHLEY, GEOFFREY, and LOWY, PETER H. Isolation of a peptide in guinea pig liver homogenate and its turnover of leucine.....	1041
BROWN, GEORGE BOSWORTH, PETERMANN, MARY L., and FURST, SIDNEY S. The incorporation of adenine into pentose and desoxypentose nucleic acids.....	1043
CHAIKOFF, I. K., LERNER, S. R., ENTENMAN, C., and DAUBEN, W. G. On the conversion of palmitic acid labeled with radioactive carbon to glucose by the alloxan-diabetic rat.....	1045
OTT, WALTHER H., RICKES, EDWARD L., and WOOD, THOMAS R. Activity of crystalline vitamin B <sub>12</sub> for chick growth.....	1047
WHITE, ABRAHAM, HOBERMAN, HENRY D., and SZEGO, CLARA M. Influence of adrenalectomy and fasting on the incorporation of isotopic nitrogen into the tissues of mice.....	1049
KORNBERG, ARTHUR. Nucleotide pyrophosphatase and triphosphopyridine nucleotide structure.....	1051
INDEX TO VOLUME 174.....	1053

### CORRECTION

On page 389, line 2, Vol. 174, No. 1, May, 1948, read " *$\delta$ -amino group*" for " *$\alpha$ -amino group*."



# CORRECTION

On page 857, title, Vol. 172, No. 2, February, 1948, read *An  $\alpha$ -Cell Hormone of the Islets of Langerhans?*



# CHANGES IN PHOSPHATE AND CARBOHYDRATE METABOLISM IN SHOCK

By E. S. GORANSON,\* J. E. HAMILTON, AND R. E. HAIST

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(Received for publication, December 15, 1947)

In recent years there have been many studies concerning the metabolic disturbances in shock secondary to injury, hemorrhage, and burns. Among the very marked changes reported are those involving carbohydrates. The glycogen stores of liver (1) and muscle are reduced in rats shocked by limb injury, and these tissues are unable to replenish their glycogen stores after glucose administration. The blood sugar levels first increase and later are reduced to normal values or below. After glucose administration by mouth the blood sugar is greatly elevated, the high level persisting for many hours. The administration of insulin reduces the blood sugar level in these animals but does not improve the storage of glycogen in the liver (1). It will be shown in this paper that insulin does not prevent reduction in the glycogen level in untraumatized muscle in the shocked rats. These observations suggest that insulin may accelerate the utilization of sugar in peripheral tissues in these animals. Sacks (2) first showed that insulin increased the incorporation of  $P^{32}$  in adenosine triphosphate (ATP) and phosphocreatine (PC) in normal anesthetized cats. In view of this finding and because of the carbohydrate changes mentioned, a comparison was made between the effect of insulin on the uptake of  $P^{32}$  in ATP and PC in shocked and in normal animals.

Some workers (3) suggest that an important factor in the development of shock is the depletion of high energy phosphorus compounds essential for the metabolism of carbohydrate. To investigate this possibility a study of the labile phosphates in the muscle of shocked rats was made with radioactive phosphorus.

## *Materials and Methods*

Rats of Wistar strain, weighing between 150 and 250 gm., were the test animals. Glycogen estimations were made by a modification of the method of Good, Kramer, and Somogyi (4). Radiophosphorus in the form of disodium phosphate was used for the phosphorus studies. Tissue samples were taken under sodium amytal anesthesia. The tissues were frozen immediately by immersion in liquid air, then finely powdered in a chilled

\* Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Toronto.

mortar, and transferred to a tared tube for weighing. The phosphates were extracted in 10 per cent trichloroacetic acid at 0°. Inorganic phosphate, phosphocreatine, and total acid-soluble phosphate fractions were determined according to the methods of Fiske and Subbarow (5) and ATP as the 7 minute-hydrolyzable phosphorus in 1 N HCl at 100°.

Aliquots of each phosphate fraction were taken for the determination of its isotopic content. The phosphorus was precipitated from the neutralized aliquot with magnesia mixture, as magnesium ammonium phosphate. Tubes were generally allowed to stand overnight, although Furchgott and Shorr (6) find that a few hours are sufficient for precipitation. Following precipitation, the tubes were centrifuged and the supernatant discarded. The precipitate was redissolved in 2 per cent HCl (1 cc.) and transferred with two washings of 1 cc. each to filter papers (7 cm.), placed on 8 inch squares of vitafilm (Haugh's Products, Toronto), and allowed to dry in air. After drying, the vitafilm was folded into envelopes and sealed with cellulose tape. The samples were then read in a Geiger-Müller counter. Estimation of the isotopic content of the phosphate sample was based on the counts per minute per mg. of phosphorus recovered in the sample. This gives a measure of the specific activity of the sample.

In the shock experiments the value for the isotope was standardized by calculating the activity on the basis of the amount of radiophosphorus injected per unit weight of the animal. The amount injected was computed from standards made up from aliquots of the preparation for injection.

The experimental rats were given 1 cc. of 25 per cent glucose by stomach tube and then were fasted for 24 hours prior to tissue sampling. Shock was induced by a clamping procedure (1). The clamps were applied to both hind legs and left in place for from 12 to 14 hours. After removal of the clamps shock began to develop.

### Results

*Muscle Glycogen*—Fasted rats were shocked as described above. In one group the animals remained in the fasting state. In a second group each rat received 2 cc. of 25 per cent glucose by stomach tube at the time of release of the clamps. A third group received 2 units of insulin per rat at the time the sugar was given. Control groups with and without sugar were also observed. Samples of muscle were removed under sodium amytal anesthesia and their glycogen content determined. Fore leg samples were obtained from both triceps muscles and hind leg samples from both gastrocnemius muscles.

In control rats the values for the glycogen content of hind leg muscle were similar to those obtained for fore leg muscle. In thirty shocked animals

the glycogen content of hind leg (injured) muscle was less than 0.05 gm. per cent, and in most cases was not measurable. The hind leg muscles were therefore not studied in the rest of the experiment.

The glycogen values for fore leg muscles in control and shocked rats are shown in Fig. 1. The glycogen content of the triceps in shocked rats is variable but usually is much less than normal. This reduction in muscle glycogen is progressive, the concentration becoming lower as shock develops.

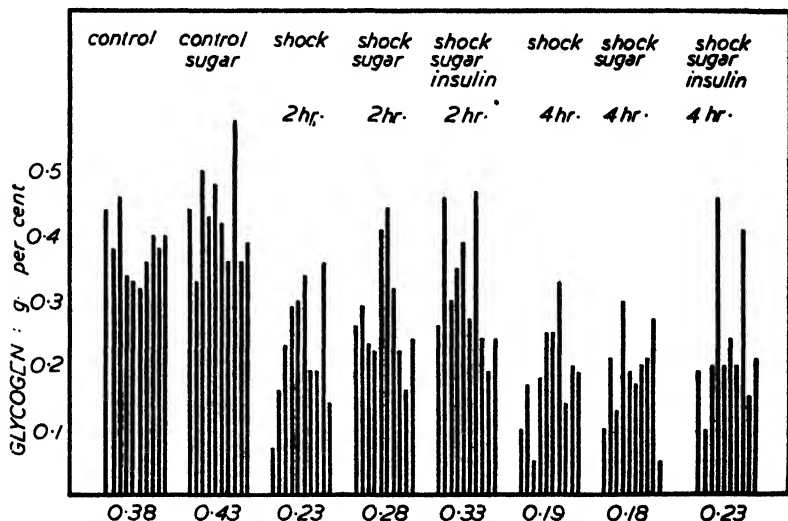


FIG. 1. Glycogen concentration in fore leg muscle (triceps) for shocked rats, shocked rats given 2 cc. of 25 per cent glucose by stomach tube at the time the clamps were removed, and shocked rats given 2 units of insulin in addition to the sugar. Values obtained early (2 hours after removal of the clamps) and late in shock (4 hours after removal of the clamps) are shown. Control values for fasting rats and for rats 4 hours after sugar administration are also given. The average values are indicated below each group.

The administration of insulin and sugar together seems to delay the glycogen reduction in triceps muscle in shock, but does not prevent it.

**Phosphorus**—Fasted rats were clamped as described above. On removal of the clamps, 2 cc. of 25 per cent glucose were given by stomach tube and at the same time radioactive phosphorus ( $P^{32}$ ), as the disodium salt, was injected subcutaneously. Those animals receiving insulin were also injected at this time with 4 units, subcutaneously.

A period of  $2\frac{1}{2}$  hours was allowed to elapse after the injections were made, in both the shocked and control groups, before tissue sampling began. In the clamped control group, the clamps were left in place during the tissue sampling. The rats were anesthetized with sodium amytal before the



samples were taken. The phosphate fractions and isotopic content of the tissue samples were determined as described previously.

The levels for the phosphorus fractions and the  $P^{32}$  values in the control animals, animals with the clamps left in position, and shocked animals are given in Fig. 2 for hind leg muscle and in Fig. 3 for fore leg muscle. It

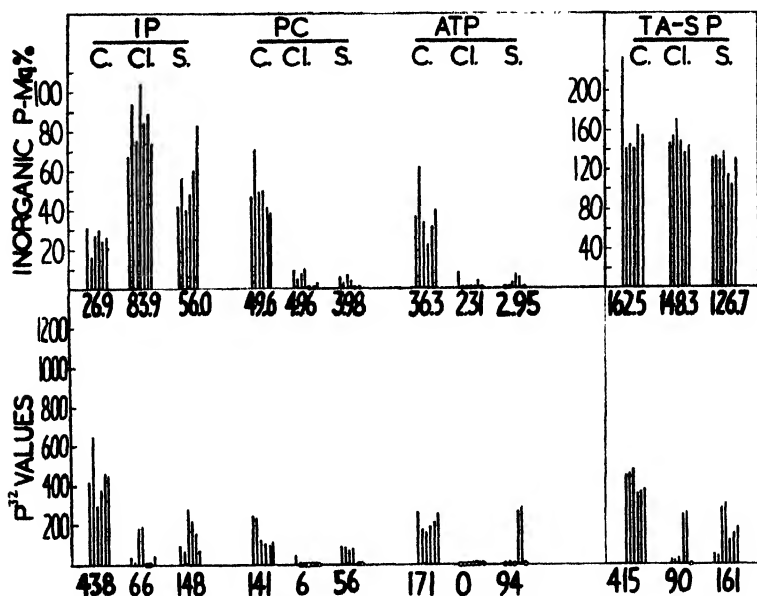


Fig. 2. Inorganic phosphorus (IP), phosphocreatine (PC), adenosine triphosphate (ATP), total acid-soluble phosphorus (TA-SP), and  $P^{32}$  values for hind leg muscle in non-clamped control rats (C.), rats with clamps left in place (Cl.), and rats in which shock developed following removal of the clamps (S.). The average values are indicated below each group. The phosphate values are expressed as mg. of inorganic phosphorus per 100 gm. of wet tissue recovered from the fraction. The  $P^{32}$  values represent the specific activity of the radioactive phosphorus present in the inorganic phosphorus recovered from each fraction, calculated on the basis of counts per minute per mg. of P recovered per million counts injected per kilo of body weight. The circles on the base-line indicate amounts not measurable.

would appear from Fig. 2 that the PC and ATP values became low in the hind limb as a result of the clamping procedure and remained low after the clamps were removed. The inorganic phosphorus of the hind leg muscle was elevated as a result of the clamping. When the clamps were removed, the values were reduced from these high concentrations but were still above the control levels. It also seems evident that the  $P^{32}$  values for these tissues were greatly reduced in all fractions as a result of the clamping and that there frequently was some increase over the low values after the clamps

were removed. The changes in phosphorus of fore leg muscle (Fig. 3) were not marked, though there is a suggestion that when the clamps are in place the PC values, both as regards the actual level and the  $P^{32}$  content, are

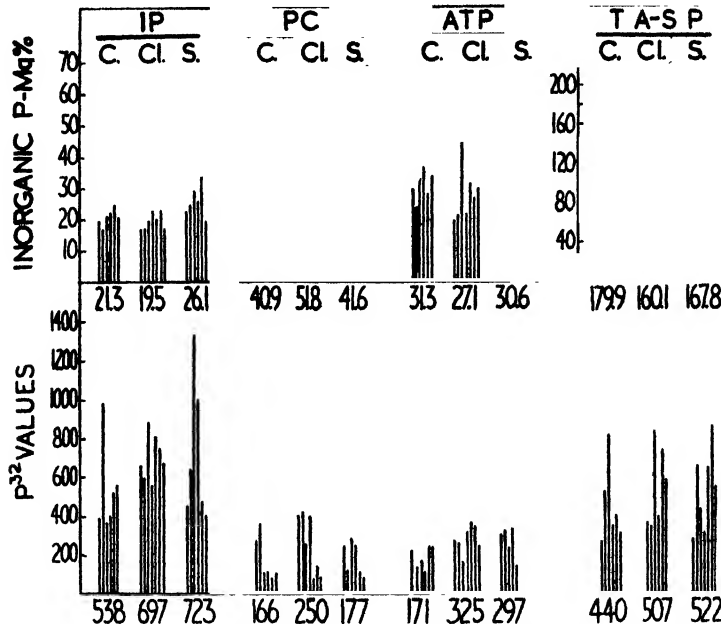


FIG. 3. Phosphate and  $P^{32}$  values for the fore leg muscles of the same group of rats shown in Fig. 2.

TABLE I

*Phosphate Values of Blood and Liver in Shocked and Clamped Rats*

		Shocked, 6 animals		Clamped controls, 7 animals		Unclamped controls, 6 animals	
		P*	Specific activity†	P	Specific activity	P	Specific activity
Blood	Inorganic P	11.33	4852	4.91	6481	4.98	6169
Liver	" "	23.28	2609	15.96	3362	18.66	2731
	Total acid-soluble P	92.2	2095	88.5	2144	85.3	1941

\* Inorganic phosphorus expressed as mg. per 100 gm. of wet tissue.

† Specific activity of the  $P^{32}$  estimated as counts per minute per mg. of P recovered per million counts injected per kilo of body weight.

elevated. Clamping also appears to elevate the  $P^{32}$  content of the inorganic phosphorus of fore leg muscle.

The inorganic phosphorus of the blood and liver and the total acid-soluble

phosphorus of the liver, together with the respective  $P^{32}$  values, are shown in Table I. The blood inorganic phosphorus values were not altered by the clamping procedure. In the shocked animals, however, the inorganic phosphorus of blood was greatly increased, though a smaller than normal proportion of this phosphorus was in the radioactive form. The total acid-soluble phosphorus and  $P^{32}$  values in the liver were of the same order of magnitude in the shocked, clamped, and control animals.

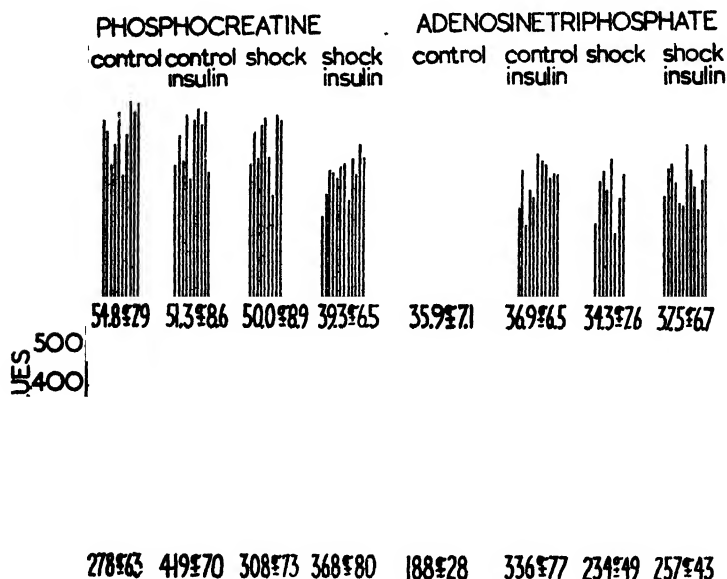


FIG. 4. The effect of insulin on ATP, PC, and  $P^{32}$  in shocked and control rats. The insulin and  $P^{32}$  were given simultaneously by subcutaneous injection 1 hour before the tissue sample was taken and, in the case of the shocked rats, 1 hour after removal of the clamps. Average values followed by the standard deviations are indicated below each group of individual values.

The effect of insulin on the PC and ATP of non-traumatized fore leg muscle of the shocked rats, and of the corresponding muscles in unclamped control rats, is shown in Fig. 4. Included in Fig. 4 also are the  $P^{32}$  values for these fractions. There was an increase in the  $P^{32}$  values of the PC and ATP fractions when insulin was given to control animals, but this was not so prominent in the shocked rats.

#### DISCUSSION

The studies concerning muscle glycogen showed that, in rats shocked by a clamping technique, insulin administration did not prevent reduction in

the glycogen concentration in the non-clamped muscle or the fall in liver glycogen, though it did cause a marked reduction in the blood sugar level in these animals. Since glycogen storage could not be entirely responsible for this reduction in blood sugar, a stimulation of glucose removal had to be considered. The results on the incorporation of  $P^{32}$  in ATP and PC after administration of insulin suggested that a part at least of the fall in blood sugar level resulted from an increased breakdown of carbohydrate.

The data show too that the damaged muscles of the clamped hind limbs of the shocked rats contained little or no PC or ATP, as was reported previously by Bollman and Flock (7), but that the uninjured fore limb muscles of these animals contained these compounds in amounts not greatly different from those found in the corresponding muscles of the non-clamped control rats. The PC of fore limb muscle in clamped animals appeared to be somewhat increased. The  $P^{32}$  values of ATP and PC in fore limb muscle were likewise not greatly altered in the shocked animals, though clamping itself caused some elevation of  $P^{32}$  in the inorganic phosphate and PC fractions. It should be pointed out that the tissue samples were obtained at a time when the liver and muscle glycogen concentrations were reduced and just prior to the time, after removal of the clamps, when the maximum number of deaths was to be expected. Under these circumstances it would seem unlikely that a depletion of energy-rich phosphate reserves in muscle or a failure in the phosphorylating mechanisms could be regarded as initiating factors in the sequence of events culminating in shock.

In these shocked rats there is a gradual diminution in the energy reserves as manifested by the steady decline in the glycogen levels in muscle and liver. This decline may be initiated by a poor oxygen supply to these tissues (8, 9).

It has been suggested by some workers (3) that the elevation of the blood inorganic phosphate in shock may reflect a breakdown of high energy phosphate at some site in the body. In the present study a breakdown of high energy phosphate in the liver or in the uninjured muscle of the shocked rats did not appear to be involved in the elevation of the blood inorganic phosphate. The results support the view expressed by Bollman and Flock that the rise in the plasma inorganic phosphate is due mainly to a diffusion of this material from the occluded hind limbs following removal of the clamps. It was observed that (a) the total acid-soluble phosphate of the traumatized muscles of the clamped controls was higher than that of the shocked rats, (b) the total acid-soluble phosphate of the liver and of the uninjured fore leg muscles of the shocked, clamped, and non-clamped groups of animals was of the same order of magnitude, and (c) the inorganic phosphate of the hind leg muscles of the shocked rats was appreciably lower than that of the clamped controls.

The findings obtained are consistent with the view that any change in the high energy compounds occurring in shock is preceded by an inadequate supply of oxygen to the tissues involved. A depletion of high energy phosphate would appear to be not an initiating factor precipitating the chain of events leading to shock, but rather the result of some other causal factor or factors.

Insulin was found to promote an increased incorporation of  $P^{32}$  in the ATP and PC of resting muscle in normal fasted rats, in agreement with finding reported by Sacks (2). An increase in the specific activity of  $P^{32}$  in ATP without an appreciable increase in the total ATP present indicates a more rapid utilization of ATP phosphate bond energy, as well as an increased rate of regeneration of the ester. While recent work has emphasized the possible action of insulin on the "hexokinase" system (10), many authors have suggested that insulin may play a catalytic rôle at some site in the carbohydrate cycle other than at the point of entry of glucose. If insulin exerts an accelerating effect on the regeneration of ATP, it may conceivably act at one or more of several levels in the degradation of carbohydrate.

When insulin was administered to the shocked rats, its effect on the  $P^{32}$  values of ATP and PC of the non-traumatized muscles was less evident. One possible explanation is that the muscle receives less insulin when its blood supply is reduced. A second possibility is that, in the presence of a reduced oxygen supply, when muscle metabolism is more anaerobic, the influence of insulin is diminished. The findings *in vitro* of Krebs and Eggleston (11), Rice and Evans (12), Shorr and Barker (13), Stadie *et al.* (14), and Stare and Baumann (15) suggest that insulin exerts a catalytic effect on the oxidation of certain constituents in the tricarboxylic acid cycle. The oxidation of a number of dicarboxylic acids has been shown to be coupled with the esterification of inorganic phosphate (16, 17). Under aerobic conditions, then, there is suggestive evidence that insulin may directly catalyze oxidative phosphorylations and thereby indirectly effect an increased phosphorylation of glucose.

#### SUMMARY

1. In the uninjured muscle of rats shocked by a clamping technique there was a decrease in glycogen concentration which was not prevented by insulin. The glycogen content of the damaged (anoxic) muscle was very low.

2. The phosphocreatine (PC) and adenosine triphosphate (ATP) content of the damaged muscle fell as a result of the clamping procedure and remained low after the clamps were removed. Inorganic phosphorus was elevated in the damaged muscle and the  $P^{32}$  values were reduced in all phosphorus fractions. After removal of the clamps the inorganic phosphorus level decreased somewhat but was still above normal. The  $P^{32}$  values,

while still low, were frequently slightly increased above the concentration found in the clamped animals.

3. The PC and ATP content of the undamaged muscle of the shocked animals did not differ from that of the non-clamped controls but the PC values were less than those of the clamped animals. The  $P^{32}$  values for PC, ATP, and inorganic phosphorus appeared to be elevated by the clamping procedure.

4. Insulin was found to promote an increased incorporation of  $P^{32}$  in PC and ATP in normal fasted controls, confirming Sacks' report (2). This effect of insulin was less evident in the non-traumatized muscle of the shocked rats.

5. A marked elevation in the blood inorganic phosphate in the shocked rats was associated with a reduction in the inorganic phosphate of the damaged muscles following removal of the clamps.

6. It is concluded that the reduction in the blood sugar level, produced by insulin in shocked rats given glucose, is due in part at least to an increased breakdown of carbohydrate. It is concluded too that the depletion of high energy phosphorus is not an initiating factor in shock. The possible involvement of insulin in oxidative phosphorylations is discussed.

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# ON THE MECHANISM OF ENZYME INHIBITION BY SULFHYDRYL REAGENTS\*

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In recent years a great deal of evidence has accumulated to show that intact —SH groups are necessary for the full activity of a variety of enzymes. The exact function of the thiol group in the protein part of these "sulfhydryl enzymes," however, remains obscure. It has been suggested that the —SH group directly participates in the activity of these enzymes, and that it might be actually located at the "active center" where combination of the enzyme and substrate takes place (1). This view is supported mainly by experiments on the protection of enzymes from the action of —SH inhibitors (reagents capable of selectively inhibiting —SH enzymes) by their substrate, prosthetic group, or analogue of the substrate (2).

If the sole function of the sulfhydryl group in these enzymes was to provide a locus for the combination of the substrate with the enzyme protein, then the inhibition of a sulfhydryl enzyme by a specific —SH reagent should be the same, regardless of the substrate used for assay, provided that the enzyme is the limiting factor in the activity determination. To the author's knowledge there are no data in the literature on this question. This paper is an account of some experiments on wheat germ lipase and D-amino acid oxidase designed to test this point.

## EXPERIMENTAL

**Methods**—Pig kidney D-amino acid oxidase was prepared essentially by the method of Krebs (3). The resulting acetone powder was extracted with 10 volumes of water at 3°, centrifuged, and the supernatant solution was lyophilized. The dried preparation was dissolved in water to give a concentration of 3 mg. per ml. immediately before use.

The lipase used in this work was prepared as follows. Unprocessed, whole wheat germ was thoroughly defatted with petroleum ether, dried, and ground in a Wiley mill. The stable dry powder was extracted with 10

\* A preliminary report of this work has been presented (*Federation Proc.*, **6**, 291 (1947)).

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volumes of ice-cold water for 15 minutes, centrifuged, and the supernatant solution was adjusted to pH 5.5 with 0.5 N acetic acid. The resulting suspension was centrifuged for 10 minutes at 3000 R.P.M., and the clear supernatant fluid was readjusted to pH 6.6 with 0.5 N  $\text{NaHCO}_3$ . After addition of saturated  $(\text{NH}_4)_2\text{SO}_4$  to 35 per cent saturation, the suspension was centrifuged, and the supernatant solution treated with more  $(\text{NH}_4)_2\text{SO}_4$  solution until 55 per cent saturation was reached. The precipitate appearing at this point was separated and preserved in the lyophilized state. Before use, it was dissolved in 0.02 M phosphate buffer, pH 6.8, dialyzed against distilled water for 2 hours, and finally subjected to 15 minutes centrifugation at 5000 R.P.M. All these operations were carried out at 3°.

Both enzymes were assayed manometrically: D-amino acid oxidase by  $\text{O}_2$  uptake and wheat germ lipase by  $\text{CO}_2$  evolution from a  $\text{NaHCO}_3\text{-H}_2\text{CO}_3$  buffer as a result of acid production. Details of the assay of this lipase have been described elsewhere (4).

*Wheat Germ Lipase*—In order to test the effect of the nature of the substrate on the inhibition brought about by sulfhydryl reagents, two conditions should be met. First, the enzyme should possess a broad enough specificity to permit the use of a series of substrates of varying structure and molecular dimensions. Second, the enzyme should satisfy all of the usual criteria applied in the detection of sulfhydryl enzymes.

In the course of experiments on the kinetics of wheat germ lipase in this laboratory, the inhibition brought about by a variety of  $-\text{SH}$  reagents was found to depend on the substrate used for assay. Since the enzyme seemed to satisfy both of the criteria mentioned above, it was decided to study its behavior toward  $-\text{SH}$  reagents in greater detail, in preference to other better known enzymes.

The enzyme acts on a wide variety of simple esters, mono- and triglycerides, and on the Tween compounds (water-soluble polyoxyalkylene sorbitan esters of long chain fatty acids; Atlas Powder Company). Although the enzyme has been obtained only in partially purified state to date, three different lines of evidence indicate that the same enzyme is involved in the activation of all of these substrates. The experimental data on this point as well as on the necessity of intact  $-\text{SH}$  groups in the enzyme for full activity have been described elsewhere (4) and will only be briefly summarized here.

With excess mono-*n*-butyric or triacetin as substrate, the highly selective  $-\text{SH}$  reagent, *p*-chloromercuribenzoate, completely and instantaneously abolished the activity of the enzyme. As would be expected, the concentration of mercurial required for this depended on the stage of purity of the enzyme preparation and on the amount of protein present;  $1 \times 10^{-3}$  to

$1 \times 10^{-4}$  M concentration effected complete inhibition. The amount of inhibitor needed for complete inactivation was thus low enough to compare favorably with established —SH enzymes, such as succinoxidase. Trivalent organic arsenicals, which have also been successfully used in testing for a large series of —SH enzymes (5, 6) produced instantaneous and complete inhibition at similar concentrations. The arsenicals and the mercurial inhibited the purified enzyme to the extent of 25 per cent or more even at  $1 \times 10^{-5}$  M concentration. The inhibition brought about by these mercaptide-forming agents was significantly reversed by addition of 10 equivalents of glutathione 20 minutes after the inhibition. *o*-Iodosobenzoate ( $1 \times 10^{-3}$  M) and ferricyanide ( $2 \times 10^{-3}$  M) strongly inhibited the enzyme, the former of the two being more effective. The lipase proved considerably less sensitive to the action of iodoacetamide, which produced only 55 per cent inhibition at  $5 \times 10^{-3}$  M concentration. The relative ineffectiveness of iodoacetamide as an —SH inhibitor has often been pointed out in the literature; established —SH enzymes, such as malic dehydrogenase, muscle adenosinetriphosphatase, and rat and pig kidney  $\delta$ -amino acid oxidase, are inert toward this reagent.

On the basis of this evidence it seems justified to conclude that wheat germ lipase requires intact thiol groups for full enzymatic activity. In this respect it may be contrasted with pancreatic lipase, in which the evidence for active sulfhydryl groups is rather weak, and the simple esterases of serum and liver, which do not require —SH groups for activity at all (6).

*Effect of Substrates on Inhibition by —SH Reagents*—While a sufficiently high concentration of *p*-chloromercuribenzoate ( $3 \times 10^{-4}$  M) inhibited the enzyme completely, for purposes of comparison an amount of the inhibitor was chosen which would produce only partial inhibition on all the substrates tested. Thus the concentration of the mercurial in the experiment described in Table I was  $1 \times 10^{-4}$  M. Under these conditions the extent of inhibition seemed to vary with the molecular size of the substrate. The longer the fatty acid residue or the alcohol, the more extensive was the inhibition. Similarly, the more esterified alcoholic hydroxyl groups in the fat molecule, the greater was the observed inhibition. Thus, for example, with tributyrin the inhibition was greatest, with tripropionin less, and with triacetin, the shortest chain fatty acid, it was least. With monobutyryl as substrate the inhibition was more extensive than with monopropionin, but less than with tributyrin. In the simple ester series this rule held equally well. From ethyl acetate to butyl acetate the inhibition increased progressively, while in another series, with methyl acetate as substrate the inhibition was even smaller than with ethyl acetate. With ethylene glycol diacetate, which has a 2-carbon alcohol and 2-carbon fatty acid groups, the

inhibition by the mercurial was the same as with ethyl acetate. The most extensive inhibition occurred with Tween 20, a water-soluble lauric acid ester having the greatest molecular dimensions in this series.

It will be noted that the concentrations of substrate varied rather widely in this experiment, the reason for this being the differences in the Michaelis constants of this lipase with respect to these substrates (4). In each case

TABLE I

*Effect of Substrate Size on Inhibition of Lipase Activity by p-Chloromercuribenzoate*

Enzyme preparation	Substrate	Activity, c.mm. CO <sub>2</sub>		Inhibition  per cent
		Control	With inhibitor	
B	Tributyrin,* 0.02 M	51.8	10.0	81
	Tripropionin,* 0.04 M	122.0	38.6	68
	Triacetin*, 0.25 M	335.9	144.4	57
	Monobutyrin, 0.112 M	58.9	18.4	69
	Monopropionin, 0.25 M	81.6	60.7	26
	Ethyl acetate, 0.45 M	73.5	61.5	16
	n-Propyl acetate, 0.20 M	81.8	53.0	35
	n-Butyl acetate, 0.10 M	73.6	31.2	58
	Methyl acetate, 0.50 M	93.3	67.5	28
	Ethyl " 0.45 "	62.6	33.9	46
	Glycol diacetate, 0.30 M	178.0	95.0	47
	Triacetin, 0.25 M	313.0	104.2	67
	Tween 20, 5%	50.3	8.3	84

Manometric measurements of acid production in bicarbonate buffer. Each Warburg vessel contained 0.6 ml. of purified wheat germ lipase containing 10 mg. of protein in Preparation A and 8.7 mg. of protein in Preparation B; *p*-chloromercuribenzoate, 0.0001 M; NaHCO<sub>3</sub>, 0.025 M; pH 7.4; total volume, 3 ml.; gas phase, 95 per cent N<sub>2</sub>-5 per cent CO<sub>2</sub>. Duration of experiments, 30 minutes; temperature, 38°. All blanks subtracted. The inhibitor was added to the enzyme 15 minutes prior to the substrate.

\* Contained 0.2 per cent gelatin; this amount of gelatin did not influence the inhibition.

the concentration of substrate was so chosen as just to saturate the enzyme and thereby give optimal activity. This was to assure that the enzyme was the limiting factor in all the experiments.

Under Preparations A and B of Table I the inhibition with any given substrate differed somewhat because of differences in enzyme concentration. Since the inhibition did not increase linearly with a decrease in enzyme concentration, a small change in the latter brought about a greater change in the per cent inhibition.

The same type of systematic variation of the inhibition was observed

when *p*-aminophenylarsine oxide, another highly specific —SH inhibitor, was used as the enzyme poison, as seen in Table II. The experimental conditions were the same as in the previous experiment with the mercurial, except that the lipase used in this experiment was a more highly purified preparation. It is apparent that again a gradually increasing inhibition was obtained with increasing molecular dimensions of the substrate in the simple ester series and in the mono- and triglyceride series. Inspection of

TABLE II

*Effect of Substrate Size and Concentration on Inhibition of Lipase by p-Aminophenylarsine Oxide ( $2.7 \times 10^{-5}$  M)*

Substrate	Activity, c.mm. CO <sub>2</sub> per 30 min.		Inhibition  per cent
	Control	With As	
Methyl acetate, 0.5 M	241.4	224.8	7
Ethyl " 0.5 "	161.0	136.1	15
Propyl " 0.1 "	118.7	88.3	26
Butyl " 0.1 "	156.4	97.6	38
Monopropionin, 0.25 M	151.6	116.2	23
" 0.5 "	175.2	130.6	25
Monobutyryl, 0.11 M	126.2	74.2	41
" 0.25 "	152.9	86.6	43
Triacetin, 0.25 M	656.3	568.7	14
Tripropionin, 0.02 M	194.7	120.9	38
Tributyryl, 0.02 M	78.0	29.8	62
Glycol diacetate, 0.25 M	483.1	425.1	12
" " 0.5 "	499.0	428.8	14

Experimental conditions as in Table I, except that 5.7 mg. of a more highly purified enzyme preparation were used in each experiment.

Table II also reveals that the reason for the different inhibitions cannot be in the differences in substrate concentration, for the concentration of substrate did not affect the extent of inhibition as seen in the case of monopropionin, monobutyryl, and glycol diacetate. Also with methyl acetate, ethyl acetate, and monopropionin, all present at the same concentration, quite different inhibitions were observed. Again, with 0.25 M glycol diacetate, monopropionin, and monobutyryl, the inhibitions differed widely.

Although displacement of the inhibitor by the substrate at higher concentrations of substrate was quite unlikely as an explanation for the varied inhibitions observed, a direct test of this point was made in the experiment

reported in Table III. In this experiment *o*-iodosobenzoate, a mild oxidizing agent, was used as an inhibitor in place of the mercaptide-forming inhibitors in which dissociation of the enzyme-inhibitor complex is appreciable. With this compound, however, oxidation of the —SH groups to disulfide linkage occurs (7), which bond could be broken only by powerful reducing agents. As is seen in Table III, when *o*-iodosobenzoate was the inhibitor the same systematic correlation was found between the nature of the substrate and the extent of the inhibition as when the mercaptide-forming reagents were used. Since none of the substrates used is capable of reducing an —SS— linkage to the thiol stage, these experiments show that displacement of the substrate cannot account for the results under discussion.

TABLE III

*Inhibition of Wheat Germ Lipase by  $1 \times 10^{-3}$  M o-Iodosobenzoate*

Substrate	Activity, c.mm. CO <sub>2</sub> per 30 min.		Inhibition  per cent
	Control	With inhibitor	
Triacetin, 0.25 M	532	266	50
Tripropionin, 0.02 M	335	108	67
Tributyryl, 0.02 M	143	30.4	89
Monobutyryl, 0.11 M	85.9	22.0	74

Experimental conditions as in Table I. 30 minutes incubation with inhibitor before addition of substrate.

Similar results were obtained when other —SH inhibitors (ferricyanide, various organic arsenoxides) were used.

**D-Amino Acid Oxidase**—The broad specificity of this enzyme is well known. The essential nature of its —SH groups for full activity has been adequately established, at least for preparations from rat and sheep kidneys (6, 8). The experiments here reported were performed with the enzyme isolated from pig kidneys, but an acetone powder of rat kidney gave entirely similar results.

Low concentrations of all mercaptide-forming —SH reagents tested inhibited the enzyme completely and instantaneously. 10 equivalents of glutathione produced complete reversal of the inhibition. A concentration of inhibitor was again selected which would produce partial inhibition only, thus permitting accurate comparison of various substrates. In the experiments recorded in Table IV,  $2.2 \times 10^{-5}$  M *p*-aminophenylarsine oxide was used for this purpose. The extent of inhibition was again independent of the concentration of the substrate, even when the latter was varied 4- to

7-fold, provided that the enzyme was nearly saturated with respect to substrate (Table IV, first and last sections). This again rules out displacement of the inhibitor by the substrate as an explanation of the findings on lipase.

With D-amino acid oxidase the inhibition was also independent of the nature of the substrate, that is, of the molecular dimensions of the amino

TABLE IV

*Effect of Substrate Size and Concentration on Inhibition of D-Amino Acid Oxidase by  $2.2 \times 10^{-5}$  M p-Aminophenylarsine Oxide*

Enzyme	Substrate <sup>a</sup>	Activity, c.mm. O <sub>2</sub> uptake per 30 min.		Inhibition
		Control	With As	
mg.				per cent
11.3	0.03 M DL-alanine	147.4	52.8	64
11.3	0.05 " "	164.4	66.0	60
11.3	0.1 " "	184.0	78.6	57
11.3	0.2 " "	199.1	82.8	59
10.2	0.05 M DL-alanine	184.2	83.8	55
10.2	0.05 " DL- $\alpha$ -aminobutyric acid	66.1	28.0	57
10.2	0.05 " DL-methionine	210.0	99.1	53
10.2	0.05 " DL-phenylalanine	115.1	56.8	51
10.2	0.05 " DL-isoleucine	162.7	71.3	56
10.2	0.05 " DL-norvaline	49.6	20.1	57
9.7	0.05 M DL-alanine	164.5	58.7	64
9.7	0.20 " "	183.9	68.3	63
9.7	0.05 " DL-valine	116.0	39.7	66
9.7	0.10 " "	133.5	46.2	65
9.7	0.094 M DL-isoleucine	170.9	66.7	61
9.7	0.10 M DL-methionine	240.0	101.9	58

Manometric measurements of O<sub>2</sub> uptake in 0.02 M pyrophosphate buffer, pH 8.3. Inhibitor added 20 minutes prior to substrate. Temperature, 38°; duration of experiments, 30 minutes; gas phase, air; total volume, 3.0 ml. The enzyme was a high speed centrifuged, lyophilized water extract of an acetone powder of pig kidney.

acid used for activity determination. The same inhibition was produced by the arsenical on alanine,  $\alpha$ -aminobutyric acid, norvaline, valine, isoleucine, methionine, and phenylalanine, although this series represents a considerable variation in molecular dimensions.

Table V shows that the oxidizing agent *o*-iodosobenzoate inhibits the enzyme to the same extent whether alanine, methionine, or phenylalanine is used as substrate, in agreement with the results obtained with the arsenical.

When the enzyme was tested on five amino acids with  $4.4 \times 10^{-5}$  M *p*-chloromercuribenzoate as the inhibitor, again essentially the same inhibition was observed in all cases. This is shown in the first part of Table VI. It will be noted that all the amino acids were present at 0.05 M concentra-

TABLE V

*Inhibition of D-Amino Acid Oxidase by  $1.33 \times 10^{-5}$  M o-Iodosobenzoate As Assayed on Various Substrates*

Substrate	Activity, c.mm. O <sub>2</sub> uptake		Inhibition  per cent
	Control	With inhibitor	
DL-Alanine, 0.05 M	167.1	36.8	78
DL-Methionine, 0.05 M	223.5	54.5	76
DL-Phenylalanine, 0.05 M	111.4	22.4	79

Experimental conditions as in Table IV. Amount of enzyme, 9.7 mg. per vessel.

TABLE VI

*Inhibition of D-Amino Acid Oxidase by p-Chloromercuribenzoate ( $4.4 \times 10^{-5}$  M) As Tested on Various Substrates*

Enzyme  mg.	Substrate	Activity, c.mm. O <sub>2</sub> uptake		Inhibition  per cent
		Control	With inhibitor	
11.3	0.05 M DL-alanine	163.7	55.2	66
11.3	0.05 " DL-valine	115.6	37.0	68
11.3	0.05 " DL-methionine	210.9	87.7	58
11.3	0.05 " DL-phenylalanine	114.7	45.4	61
11.3	0.05 " DL-isoleucine	161.5	76.9	53
10.0	0.05 M DL-methionine	156.7	17.0	89
10.0	0.116 " "	179.5	37.5	79
11.3	0.05 M DL-alanine	164.5	54.1	67
11.3	0.2 " "	183.9	92.4	50
11.3	0.05 " " + 0.15 M β-alanine	164.5	65.5	60
11.3	0.05 " " + 0.15 " glycine	147.5	58.6	60

Experimental conditions as in Table IV.

tion. With isoleucine the inhibition was slightly less in this particular experiment, but this probably represents an experimental error.

The experiments reported in the second and third sections of Table VI were designed to demonstrate further that inhibition is independent of sub-

strate concentration. When the concentration of methionine was more than doubled or the amount of  $\alpha$ -alanine increased 4-fold, the inhibition decreased only 10 to 20 per cent. It will be remembered that with the arsenicals as inhibitors even this small effect did not occur. The apparent explanation of this slight discrepancy is that the mercurial, unlike the arsenoxide, is capable of combining with the carboxyl groups of amino acids when the latter are present in very large concentration. However, the affinity of *p*-chloromercuribenzoate for the —SH groups of D-amino acid oxidase is many thousand times greater than for the —COOH group of the amino acid.

In order to show that the small but consistent decrease in the inhibition brought about by delayed addition of excess amino acids could be ascribed to a non-specific combination with the —COOH group rather than dissociation of the mercaptide by a specific substrate, the following experiment was performed. Two carboxylic acids were selected with the same pK value as  $\alpha$ -alanine which, however, are not attacked by the enzyme. Glycine and  $\beta$ -alanine were used for this purpose. In the last section of Table VI it is seen that when these amino acids were used to provide the high —COOH concentration essentially the same type of small decrease in inhibition was observed as with excess  $\alpha$ -alanine.

Thus it seems justified to conclude that the inhibition of D-amino acid oxidase by both arsenicals and mercurials is independent of the concentration of substrate and that displacement of the inhibitor by the specific substrate does not occur to a significant extent.

#### DISCUSSION

An interpretation of the experimental observations on the inhibition of wheat germ lipase reported in this paper will now be attempted. It is clear that the different inhibitions found when esters of varying structure were used as substrates are valid only if the enzyme was the limiting factor in all instances. Considerable effort was made to test this point. Varying the substrate concentration, as noted above, or the pH of the assay, in no way affected the differential inhibition observed. All evidence pointed to the fact that the enzyme protein was actually the limiting factor in all these experiments.

The possibility of some reversal of the inhibition by substrates has already been referred to in the experimental part. This point was especially carefully tested in view of the known fact that with enzymes like succinoxidase the substrate may protect the enzyme from various —SH inhibitors. That displacement of the inhibitor by the substrate occurred to no measurable extent is evident from Table II and this is, of course, eliminated by experiments with oxidizing agents, such as are reported in Table III.



Furthermore, it should be pointed out that the dissociation constants of the lipase for most of these substrates have been experimentally determined (4) and no positive correlation was found with the extent of inhibition produced by any —SH reagent when these esters were used as substrates. As a matter of fact, in most instances, there was a negative correlation. The smaller the dissociation constant of the enzyme for the substrate, the more tightly it binds that substrate, and hence the more easily the latter should relieve an inhibition if displacement does occur. Actually, the substrates with the smallest dissociation constant (tributyryl, tripropionin) were the very ones which allowed the greatest inhibition by —SH reagents.

The data on D-amino acid oxidase are compatible with the idea that the —SH groups of that enzyme may be directly involved in the activation of its substrates. On the other hand, the demonstration that the effect of any —SH reagent on wheat germ lipase depends on the particular substrate used for assay, and further that complete inhibition toward one substrate can be demonstrated with little effect toward other substrates at the same concentration of inhibitor indicates that the —SH groups of this lipase are not directly involved in its activity.

The apparent correlation found between the molecular dimensions of the substrate and the inhibition of the lipase suggests further possibilities. It seems entirely possible that not only one or two functional groups located at the active center but that also the configuration and arrangement of the amino acids at some distance from the active center might determine the catalytic activity of enzymes. Thus if the —SH groups of wheat germ lipase were located in the vicinity of, or at some distance from, the active center, without being directly involved in the activation of the substrate, it is conceivable that formation of complexes or oxidation of these —SH groups, as in the foregoing experiments, might *sterically* interfere more with the approach of the larger substrate molecules to the active surface than with that of smaller ones. This might then account for the influence of the molecular dimensions of the substrate on the inhibition observed.

It would be interesting to extend these findings to other —SH enzymes. There are, unfortunately, few enzymes known that would satisfy both of the criteria listed in the experimental part. Possibly crystalline papain, an established —SH enzyme, might be used for this purpose, provided that an adequate number of synthetic substrates are available. It would also seem desirable to examine "essential" amino or phenolic groups with the same view in mind.

A further corollary of these findings seems to be that it is no longer quite safe to conclude that two or more enzymes with similar functions are present in a preparation on the basis of different inhibitions obtained with two or more substrates for assay.

## SUMMARY

1. The extent of inhibition of wheat germ lipase by sulfhydryl reagents (arsenicals, *p*-chloromercuribenzoate, oxidizing agents) varies with the particular substrate used for assay.

2. The variation seems to be well correlated with the molecular dimensions of the substrate. The larger the substrate molecule, the greater the inhibition produced by any —SH reagent.

3. Reversal of the inhibition by higher substrate concentrations has been eliminated as a possible explanation of these observations.

4. The data have been interpreted as suggesting a possible steric interference with the approach of substrates to the active surface of the lipase by —SH reagents.

5. The inhibition of D-amino acid oxidase by —SH reagents is independent of the nature of the substrate used for assay, in agreement with the idea that the —SH groups of this enzyme might be directly involved in the activation of substrates.

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# THE IMMUNOCHEMISTRY OF TOXINS AND TOXOIDS

## V. THE SOLUBILITY OF STAPHYLOCOCCAL TOXIN IN METHANOL-WATER MIXTURES UNDER CONTROLLED CONDITIONS OF PH, IONIC STRENGTH, AND TEMPERATURE\*

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The success encountered in the purification of tetanal toxin (1) and toxoid (2, 3), of diphtherial toxoid (4), and of soluble pertussal antigens (5) warranted attempts to purify other toxins and toxoids by the multiphase system of protein fractionation. The system employs the precise adjustment of methanol concentration, pH, ionic strength, and protein concentration under rigidly controlled conditions of temperature in order to separate or isolate proteins.

Previous attempts to purify and concentrate staphylococcal toxin have met with varying degrees of success. Holt (6) employed ammonium sulfate to concentrate staphylococcal toxin. Burnet and Freeman (7) precipitated the toxin with acetic acid, while Boivin and Izard (8) used trichloroacetic acid. It is the object of the present communication to report studies on the solubility of staphylococcal toxin in methanol-water mixtures. Subsequent publications will deal with the actual isolation and characterization of the toxin.

### Materials

*Staphylococcus aureus*, strain Wood No. 46, was grown in a liquid medium containing inorganic salts of sodium, potassium, calcium, and magnesium, ammonium lactate, and proteose-peptone. Cultures were incubated for 24 to 48 hours, during which time they were aerated with a gas mixture of 20 per cent carbon dioxide and 80 per cent oxygen. The cell-free filtrates of the cultures contained the  $\alpha$ -toxin.<sup>1</sup>

White, Swiss, female mice<sup>2</sup> weighing about 20 gm. each were used for determination of minimal lethal dose of the toxin.

All reagents were either C.P. or the best grade obtainable. Dilutions of toxin for m.l.d. determinations were made in 1 per cent Witte's peptone in saline.

\* Aided by a grant from the Lederle Laboratories Division, American Cyanamid Company.

<sup>1</sup> We wish to express our gratitude to Dr. H. D. Piersma and Mr. J. N. Adam, Jr., of Lederle Laboratories for furnishing the staphylococcal toxin used in this work.

<sup>2</sup> Obtained from Tumblebrook Farms, Brant Lake, New York.

## Methods

### Immunological

*Flocculation Test*—The procedure for the Lf test was essentially the same as that described by Pillemer (2). Antitoxin diluted to 100 units per ml. with M/15 phosphate buffer of pH 7.4 was added in increasing amounts to a series of serological test-tubes. Crude or purified toxin in 2.0 ml. amounts was then added to each tube and the volume brought to a total of 2.5 ml. with phosphate buffer. After the contents were thoroughly mixed, the tubes were placed in a water bath at 50°. The tube in which the first definite flocculation occurred was the indicator tube, and the time (in minutes) required for flocculation was the Kf. In certain experiments, the number of units of antitoxin was kept constant, and the amount of toxin was varied. This made comparison of various Kf values possible.

*Minimal Lethal Dose*—The method described by Pillemer (2) for the determination of m.l.d. was followed. Mice were injected intraperitoneally with 1.0 ml. of varying dilutions of toxin. Mice were observed for 96 hours following the injection; however, death usually occurred within 24 hours and rarely after 48 hours. The end-point chosen was that dilution which killed all of the mice injected within 96 hours.

It will be noted in Tables I to V that flocculating capacity and lethal activity of the toxin are not directly related. The true relationship of these two tests cannot be determined until the toxin has been obtained in pure form. However, it should be pointed out that complete recovery of Lf units associated with a slight loss in m.l.d. units might indicate the presence of toxoid, which, if it were present, would retain the ability to flocculate but would, of course, not be detected in the m.l.d. test. Since it was difficult to obtain reproducible results in the m.l.d. test and since the Lf test, in contrast, was highly reproducible, the latter was relied on in this work for estimating yield and purity. The m.l.d. test was used only to detect the presence of lethal activity after each purification procedure. The actual yield of m.l.d. units was not significant in determining the optimal conditions of fractionation so long as at least 50 per cent of the lethal activity could be detected. The Lf value of the toxin as obtained by the flocculation test compared favorably with the  $L_+$  value obtained by the lethal test and with the Lh value obtained by the hemolytic test. The neutral mixture of toxin and antitoxin as determined by the Lf test did not kill mice or hemolyze red cells.

### Chemical

Determination of nitrogen was made in duplicate by the micro-Kjeldahl method of Pregl. Ionic strength was determined by calculation after the

addition of all reagents to the system. Hydrogen ion concentrations were determined on the glass electrode.

## EXPERIMENTAL

Precipitation of staphylococcal toxin was studied under varying conditions of pH, methanol concentration, ionic strength, protein concentration,

TABLE I

*Precipitation of Staphylococcal Toxin Lot 80B45 under Varying Conditions of pH, Methanol Concentration, Ionic Strength, and Protein Concentration at  $-5^{\circ}$*

Sample No.	pH	Methanol	Ionic strength	Protein	N per ml.	Lf per ml.	Kf	M.l.d. per ml.	Lf per mg. N	Yield	
										Lf	M.l.d.
		per cent		per cent	mg.		min.			per cent	per cent
4	7.0	40	0.08	1.0	0.176			0.5			1
12	6.5	25	0.10	1.3	0.073			0.5			1
3	6.0	40	0.08	1.0	0.234						
2	5.0	40	0.08	1.0	0.269	12	110	5.0	45	73	10
8	4.6	40	0.08	1.0	0.310	14	50	50.0	45	82	100
7	4.3	40	0.08	1.0	0.277	16	45	50.0	58	94	100
11	4.3	25	0.10	1.3	0.135	17	28	25.0	126	100	50
1	4.0	40	0.08	1.0	0.203	16	45	25.0	79	98	50
6	4.0	40	0.08	1.0	0.221	15	45	50.0	68	88	100
10	4.0	25	0.10	1.3	0.109	17	28	25.0	156	100	50
16	4.0	20	0.11	1.3	0.081	17	28	50.0	210	100	100
17	4.0	15	0.11	1.4	0.060	17	24	38.0	284	100	75
18	4.0	15	0.17	1.4	0.044	15	25	50.0	341	88	100
23	4.0	15	0.11	1.4	0.038	13	20	50.0	342	84	100
9	4.0	10	0.12	1.5	0.049	14	28	12.5	286	82	25
5	3.7	40	0.11	1.0	0.206	15	50	50.0	73	88	100
13	3.0	7	0.19	1.6	0.047	12	180	5.0	255	76	10
14	2.0	4	0.23	1.6	0.040			1.0			2
15	1.5	2	0.30	1.6	0.038						
Parent toxin, Lot 80B45 . . . . .					3.033	17	40	50	6		

and temperature. Aliquots of parent staphylococcal toxin at pH 7.45 were cooled to  $0^{\circ}$ , and ice-cold acetic acid, in various concentrations and in constant volume, was added to give hydrogen ion concentrations between pH 4.0 and 7.0. pH values from 1.5 to 3.7 were obtained by the addition of cold HCl. The samples were cooled in a bath at  $-5^{\circ}$  to the freezing point of the mixtures. Calculated amounts of methanol previously chilled to  $-20^{\circ}$  were slowly added with stirring. The mixtures were allowed to stand at  $-5^{\circ}$  overnight, centrifuged<sup>3</sup> at  $-5^{\circ}$ , and the precipitated material dis-

<sup>3</sup> An International PR-1 refrigerated centrifuge was used throughout these experiments.

solved at 0° to the original volume of the parent toxin in M/15 phosphate buffer of pH 7.4. Nitrogen, Lf, and m.l.d. determinations were carried out on the dissolved precipitates. Yields were calculated in terms of recovery of Lf or m.l.d. units from the parent toxin.

Staphylococcal toxin was most satisfactorily precipitated at pH 4.0 in the presence of 15 per cent methanol at ionic strengths of 0.11 to 0.17 (Table I). Increasing the methanol concentration at this hydrogen ion concentration resulted in precipitation of more extraneous nitrogenous constituents along with the toxin as indicated by the decrease in Lf per mg. of N. Decreasing the methanol concentration below 15 per cent resulted in incomplete precipitation of toxin. Hydrogen ion concentrations as low as pH 4.6 or as high as pH 3.7 in 40 per cent methanol also precipitated toxin in good yield, but the purity of these products was greatly decreased, and the

TABLE II

*Influence of Ionic Strength on Precipitation of Staphylococcal Toxin at pH 4.0, Methanol Concentration of 15 Per Cent, and Temperature of -5°*

Sample No.	Ionic strength	Protein	N per ml.	Lf per ml.	Kf	Lf per mg. N	Yield of Lf
		<i>per cent</i>	<i>mg.</i>		<i>min.</i>		<i>per cent</i>
19	0.06	0.8	0.087	17	30	195	99
20	0.10	1.2	0.059	16	32	271	92
21	0.15	1.2	0.062	14	37	226	83
22	0.20	1.2	0.057	14	40	246	83
Parent toxin, Lot 80B45....			3.033	17	40	6	

Kf was increased. Hydrogen ion concentrations greater than pH 3.7 are injurious to the toxin, even at low methanol concentrations. Hydrogen ion concentrations less than pH 5.0 at methanol concentrations as great as 40 per cent do not precipitate the toxin. Precipitation at pH 4.0 in 15 per cent methanol yields toxin that is 50 times more pure on a nitrogen basis than the parent toxin.

The effect of an increase or decrease in ionic strength at pH 4.0 and 15 per cent methanol on the solubility of the toxin is shown in Table II. A greater degree of purification with a good yield was obtained when the ionic strength was adjusted to approximately 0.1 than when higher or lower salt concentrations were employed.

Staphylococcal toxin which was precipitated at pH 4.0, methanol concentration of 15 per cent, ionic strength of 0.11, and temperature of -5° was dissolved in 0.15 M sodium acetate to one-twentieth of the volume of the parent toxin and designated Fraction P-I. Aliquots of Fraction P-I di-

luted with an equal part of ice-cold distilled water were placed at 0°, and various concentrations of acetic acid in constant volume were added to give pH values from 3.6 to 5.9. Before the addition of methanol, the samples were chilled to the freezing point of the mixtures in a -5° bath. These samples were allowed to stand at -5° overnight, while those samples which did not contain methanol were held at 0°. The precipitates were then re-

TABLE III

*Precipitation of Fraction P-II from Fraction P-I\* under Varying Conditions of pH, Methanol Concentration, Ionic Strength, Protein Concentration, and Temperature*

Sample No.	pH	Methanol	Ionic strength	Protein	Temperature	N, insoluble	Lf. insoluble	Kf	M.l.d., insoluble	Lf per mg. N	Yield	
											Lf	M.l.d.
		per cent		per cent	°C.	mg.		min.			per cent	per cent
12	5.5	40	0.04	0.13	-5	3.11	1060	13	2650	341	83	100
6	5.5	0	0.07	0.22	0	1.53						
11	5.0	25	0.05	0.17	-5	3.41	1113	12	2650	326	87	100
5	5.0	0	0.07	0.22	0	2.27	636	21	1060	280	50	40
4	4.6	0	0.07	0.22	0	2.46	1166	13	2650	474	92	100
2	4.4	0	0.07	0.25	0	2.64	1113	15	2650	422	75	100
10	4.3	20	0.06	0.18	-5	3.47	1325	13	2650	382	100	100
9	4.3	10	0.07	0.20	-5	3.20	1272	12	2650	397	100	100
8	4.3	0	0.07	0.22	0	2.08	1166	13	2650	561	92	100
3	4.1	0	0.07	0.22	0	2.00	954	17	2120	476	75	80
7	4.0	10	0.07	0.20	-5	2.62	1166	14	2650	446	92	100
1	4.0	0	0.07	0.25	0	2.16	1060	15	2650	490	71	100
17	3.6	0	0.07	0.22	0	1.30						

\* All samples, except Samples 1 and 2, were derived from Fraction P-I which contained 0.76 mg. of N per ml., 240 Lf per ml., 316 Lf per mg. of N, 500 m.l.d. per ml., and had a Kf of 20 minutes. Samples 1 and 2 were derived from Fraction P-I which contained 0.87 mg. of N per ml., 280 Lf per ml., 322 Lf per mg. of N, 500 m.l.d. per ml., and had a Kf of 20 minutes.

moved by centrifugation at either -5° or 0°, depending on the methanol concentration.

The precipitates were dissolved at 0° in M/15 phosphate buffer of pH 7.4 to one-tenth the volume of the parent toxin. The dissolved precipitates (Fraction P-II) (Table III) and certain supernatant fluids (S-II) (Table IV) were tested for nitrogen content and Lf and m.l.d. values. Yields were calculated on the recovery from Fraction P-I.

The conditions for attaining the greatest purity without appreciable loss of toxic or flocculating activity were pH 4.3, ionic strength of 0.07, protein concentration of 0.22 per cent, at 0° (Table III). In the absence of meth-



anol, hydrogen ion concentrations greater or less than pH 4.3 did not increase the yield of toxin and generally decreased the purity. When the pH was lowered to 4.0 or raised to 5.0, methanol was necessary for satisfactory

TABLE IV

*Separation of Fraction S-II from Fraction P-I\* at 0° in Absence of Methanol, under Varying Conditions of pH, Ionic Strength, and Protein Concentration*

Sample No.	pH	Ionic strength	Protein	N, soluble	Lf, soluble	Kf	M.l.d., soluble	Lf per mg. N	Yield	
									Lf	M.l.d.
			per cent	mg.		min.			per cent	per cent
20	5.9	0.04	0.11	3.33	1165	28	2330	350	86	83
19	5.6	0.04	0.11	3.01	1165	28	1165	387	86	41
18	5.6	0.02	0.06	4.12	1419	25	2365	344	100	81
6	5.5	0.07	0.22	2.77	1100	20	2260	397	86	85
22	5.4	0.04	0.11	2.77	979	23	2330	353	72	83
21	5.1	0.04	0.11	2.21	583	27	1165	264	43	41

\* Fraction P-I contained 0.76 mg. of N per ml., 240 Lf per ml., 316 Lf per mg. of N, 500 m.l.d. per ml., and had a Kf of 20 minutes.

TABLE V

*Extraction of Fraction P-II\* at Various Hydrogen Ion Concentrations with Phosphate or Acetate Buffers of 0.15 Ionic Strength*

Sample No.†	pH	N extracted	Lf extracted	Kf	M.l.d. extracted	Lf per mg. N	Yield ‡	
							Lf	M.l.d.
		mg.		min.			per cent	per cent
30	6.5	2.74	1400	15	3500	511	75	100
29	6.0	2.48	1708	15	3500	688	92	100
28	5.5	1.85	1400	12	3500	756	75	100
27	5.0	1.16	1400	10	2800	1207	75	80

\* Fraction P-II was separated from Fraction P-I at pH 4.3, ionic strength of 0.05, protein concentration of 0.26 per cent, at 0° in the absence of methanol.

† Samples 30 and 29 were extracted with phosphate buffers and samples 28 and 27 were extracted with acetate buffers at the pH indicated.

‡ Yields were based on recovery from Fraction P-I which contained 0.87 mg. of N per ml., 280 Lf per ml., 322 Lf per mg. of N, 500 m.l.d. per ml., and had a Kf of 20 minutes.

recovery of toxin, but this also resulted in precipitation of non-specific nitrogenous material. In fact, the addition of methanol at any pH resulted in the precipitation of impurities. All efforts to maintain the toxin in a soluble state while precipitating the non-toxic constituents of Fraction P-I were less satisfactory (Table IV) than the above procedures. Increasing

the ionic strength at pH 4.3 resulted in increased solubility of toxin as well as of non-specific nitrogenous products.

The precipitate obtained by fractionation of Fraction P-I at pH 4.3, ionic strength of 0.07, protein concentration of 0.22 per cent, at 0° was designated P-II. This material was extracted with buffers of various hydrogen ion concentrations and ionic strengths. The suspensions were stirred for 5 minutes and allowed to stand for 10 minutes. The precipitates were then removed by centrifugation. Extraction and centrifugation procedures were carried out at 0°. The supernatant fluids were analyzed for toxin and nitrogen content. Yields were based on the recovery from Fraction P-I.

It will be seen in Table V that extraction with acetate buffer of pH 5.0 and ionic strength of 0.15 was superior to extraction with acetate buffers of pH 5.5 or phosphate buffers of pH 6.0 and 6.5 of ionic strength 0.15. Although a slight loss in yield resulted from extraction at pH 5.0, the purification obtained outweighed the loss. In other experiments it was found that buffers of ionic strengths greater or less than 0.15 were less effective extracting agents.

The procedure reported above yields a toxin having over 1200 Lf per mg. of N and a purification factor<sup>4</sup> of 200. On the basis of the foregoing results, large quantities of toxin will be processed in order that sufficient quantities of this material may be available for the actual isolation of the toxin.

#### SUMMARY

The solubility of staphylococcal toxin in methanol-water mixtures of controlled pH, ionic strength, and temperature has been determined. Staphylococcal toxin is precipitated from the parent filtrate at pH 4.0, methanol concentration of 15 per cent, ionic strength of  $0.11 \pm 0.01$ , and temperature of -5°. Reprecipitation at pH 4.3 in the absence of methanol, at 0°, followed by extraction of the precipitate with acetate buffer of pH 5.0 and ionic strength of 0.15, yields toxin which has over 1200 Lf per mg. of N and retains its lethal activity.

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<sup>4</sup> The purification factor is defined as (Lf per mg. of N of purified toxin)/(Lf per mg. of N of parent toxin).



# INHIBITION OF HYALURONIDASE ACTION BY DERIVATIVES OF HESPERIDIN

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In a previous communication (1) it was reported that certain vitamin P compounds exerted an inhibitory action on the breakdown of hyaluronic acid by testis hyaluronidase, as determined turbidimetrically. This action was manifested for the most part only when the compounds in question were combined with ascorbic acid, and only when their concentration was relatively high (0.1 mg. per cc.). Since the original object of the investigation was its application to the field of capillary fragility, it was considered to be of importance to attempt an activation of the hyaluronidase-inhibiting properties manifested by vitamin P compounds, without destroying their vitamin P specificity.

Heparin has been found to cause a marked inhibition of hyaluronidase activity at very low concentrations (2). This action depends on the sulfate groups present in the molecule, for it has been shown that when heparin is desulfonated it no longer functions as an inhibitor of hyaluronidase (2). Heparin has no vitamin P specificity, but these findings suggested that a potentiation of the action of vitamin P compounds in inhibiting the breakdown of hyaluronic acid by hyaluronidase might be accomplished by the formation of suitable derivatives.

Hesperidin was chosen as a representative vitamin P compound. The demonstrated importance of the sulfate groups in heparin suggested the synthesis of a sulfonated hesperidin. It has been shown that phosphate groups may potentiate the action of inhibitors on enzyme systems (3); accordingly a phosphorylated hesperidin was synthesized. Finally, hesperidin was acetylated to form a representative organic derivative.

The methods employed for the synthesis of these compounds are presented in detail below. In each case the starting material was hesperidin which had been purified by treatment with formamide.

## EXPERIMENTAL

*Acetylated Hesperidin*—35 gm. of hesperidin were suspended in 350 cc. of glacial acetic acid. A few cc. of concentrated  $H_2SO_4$  were added slowly with stirring. The mixture was placed on the steam bath and heated with occasional stirring for about 20 minutes. At the end of this time a dark red solution formed. This was allowed to cool to room temperature and

treated with 5 volumes of water. The precipitate which formed was filtered off after standing for several hours in the ice box and washed with water.

A yield of 20 gm. of a dark yellow material was obtained. The substance was insoluble in water, and freely soluble in alcohol. It was purified by solution in 100 cc. of alcohol, and reprecipitation by the addition of 10 volumes of water. A few cc. of acetic acid were added to aid in the formation of a precipitate. The precipitate was filtered off and washed with water. Yield, 11 gm.; m.p., 120°.

Analysis showed the material to have an acetyl content of 13.2 per cent. The calculated acetyl content for the diacetate of hesperidin is 12.1 per cent; for the triacetate, 17.1 per cent. The material is thus a mixture, with the diacetate predominating.

*Sulfonated Hesperidin (Na Salt)*—To 40 gm. of hesperidin were added 100 cc. of  $\text{H}_2\text{SO}_4$  slowly and with cooling. A dark red solution formed, which was poured slowly and with cooling into 500 cc. of alcohol. The solution was neutralized with saturated NaOH, and the precipitated  $\text{Na}_2\text{SO}_4$  was filtered off. The precipitate was washed on the filter with alcohol and the washings added to the filtrate. The combined solutions were made alkaline with NaOH and poured into 500 cc. of alcohol. The orange-colored precipitate which formed was filtered off, washed with alcohol, and dried.

Purification was accomplished by solution in a minimum volume of water and reprecipitation with 5 volumes of alcohol. Yield, 27 gm. The melting point was indeterminate, the compound decomposing gradually over a wide temperature range.

Analysis showed a sulfur content of 6.3 per cent. The calculated content for the monosulfonated hesperidin Na is 4.4 per cent; for the disulfonated, 7.8 per cent. The material is thus a mixture of the mono- and disulfonates.

*Phosphorylated Hesperidin*—37 gm. of hesperidin were suspended in 400 cc. of anhydrous pyridine. 100 cc. of  $\text{POCl}_3$  were added slowly, with stirring. Heat was evolved, and a dark brown solution formed. This was allowed to cool to room temperature, and a bulky precipitate settled out. Water was added cautiously with cooling until there was no further reaction and the precipitate was dissolved. The solution was filtered to remove any insoluble particles and then poured into 5 volumes of alcohol. The precipitate which formed was filtered off and washed with alcohol. Purification was accomplished by solution in a minimum volume of water and reprecipitation with 5 volumes of alcohol. Yield, 33 gm. The melting point was indeterminate, the compound decomposing gradually over a wide temperature range.

Analysis showed a phosphorous content of 14.5 per cent. The calculated

content for the pentaphosphate is 15.7 per cent; for the tetraphosphate, 13.7 per cent. The material is thus a mixture of the penta- and tetraphosphates of hesperidin.

Determinations of the effect of the various compounds on hyaluronidase activity were made turbidimetrically by the method previously reported (1). The hyaluronidase used was prepared from bull testes by the method of Kass and Seastone (4). Hyaluronic acid was prepared from bovine vitreous humor by the method of Seastone (5).

In addition to the hesperidin derivatives, determinations were made on suramin (germanin, Bayer 205) and on salmine sulfate. Combinations of salmine sulfate and both sulfonated and phosphorylated hesperidin and ascorbic acid and acetylated and sulfonated hesperidin were tested.

TABLE I  
*Inhibitory Effect of Various Compounds on Hyaluronidase Action*

Substance	Inhibition			
	1000 $\gamma$ per cc.	100 $\gamma$ per cc.	10 $\gamma$ per cc.	1 $\gamma$ per cc.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Hesperidin.....	0	0		
Acetylated hesperidin.....		20		
Sulfonated ".....		85	25	
Phosphorylated hesperidin.....		80	65	0
Suramin.....		85	75	15
Salmine sulfate.....	0	0		

### Results

The sulfonated and phosphorylated hesperidins proved to be extremely potent inhibitors of hyaluronidase. Suramin was found to be even more effective, while salmine sulfate was without activity. These results are presented in Table I. Results previously obtained with hesperidin are included to provide a standard of comparison.

Salmine sulfate, while without effect on hyaluronidase, was found completely to neutralize the inhibition caused by both sulfonated and phosphorylated hesperidin. As may be seen from Table I, sulfonated hesperidin at a concentration of 100  $\gamma$  per cc. caused an inhibition of 85 per cent, phosphorylated hesperidin one of 80 per cent. When either of these substances was combined with salmine sulfate, however, at a concentration of 100  $\gamma$  per cc., no inhibition at all was found.

It had previously been reported (1) that, with both at concentrations of 100  $\gamma$  per cc., hesperidin caused a potentiation of 100 per cent in the inhibitory action of ascorbic acid on hyaluronidase. This was found to be the

case also with acetylated hesperidin. Because of the large inhibition caused by sulfonated and phosphorylated hesperidin at 100  $\gamma$  per cc., these could not be tested at this concentration in combination with ascorbic acid. Sulfonated hesperidin was tested at 10  $\gamma$  per cc. with ascorbic acid. At this concentration no potentiation of the inhibitory effect of the ascorbic acid was found.

#### DISCUSSION

The results obtained show that the formation of derivatives of hesperidin can result in a great potentiation of its ability to inhibit the action of hyaluronidase on hyaluronic acid. Hesperidin was originally chosen for the investigation as a representative vitamin P compound, and it seems probable that the formation of similar derivatives of any member of the vitamin P group would result in the same potentiation.

It is true that inhibition of hyaluronidase action has been demonstrated by many compounds which show no vitamin P activity *in vivo*. The action of such substances on hyaluronidase *in vitro*, however, differs in one respect from that of the vitamin P compounds: they cause no potentiation of the inhibitory effect on hyaluronidase of ascorbic acid. It can therefore be postulated (on the basis of necessarily incomplete studies) that the property of potentiating the action of ascorbic acid on hyaluronidase *in vitro* is a measure of vitamin P specificity.

If, as has been suggested (6), hyaluronidase plays a rôle "in accentuating capillary fragility" the importance of an effective inhibitor of hyaluronidase in the clinical treatment of this condition is manifest. It would appear obvious, however, that such a substance must have a vitamin P specificity; heparin, for example, although an extremely effective hyaluronidase inhibitor, would be useless in this connection because of the other physiological effects which it is known to produce.

The importance of the derivatives of hesperidin which have been synthesized and tested, then, lies in the fact that they exert a very much more powerful inhibitory effect on hyaluronidase than do the vitamin P compounds themselves, while retaining their vitamin P specificity, as evidenced in the case of the acetylated hesperidin. A more specific evaluation of the utility of these compounds must of course await testing *in vivo*.

#### SUMMARY

Three derivatives of hesperidin have been synthesized. These include acetylated hesperidin, sulfonated hesperidin, and phosphorylated hesperidin.

All three have been shown to exert a greater inhibitory effect on hyaluronidase action than does hesperidin itself. In the case of the acetylated hes-

peridin, the same potentiation of the action of ascorbic acid as was evidenced by hesperidin has been demonstrated.

The inhibitory effect of the phosphorylated hesperidin and the sulfonated hesperidin has been shown to be neutralized by salmine sulfate.

Suramin has been shown to be an effective inhibitor of hyaluronidase action.

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# THE ASSAY AND DISTRIBUTION OF COENZYME A\*

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This is the first paper in a series dealing with partial identification of coenzyme A, a pantothenic acid derivative (1), and with the enzymatic reactions in which this coenzyme is involved. These studies originated with the observation that pigeon liver extract on autolysis lost the ability to acetylate sulfanilamide (2). Such ability, however, was regained fully on addition of boiled extract of liver and of other organs. This observation characterized the enzymatic acetylation of aromatic amines, with adenosine triphosphate as a condensing agent, as being due to an apoenzyme-coenzyme system. The ease with which coenzyme was destroyed by autolysis suggested a convenient method of preparation of the apoenzyme which proved valuable for coenzyme evaluation. In the present paper, the assay procedure will be described. The results of assays for coenzyme A in various materials show a ubiquitous distribution of coenzyme A in living cells.

## *Preparation of Pigeon Liver Extract*

It has been mentioned in a previous publication (2) that the most suitable extracts were obtained from acetone powder. An important advantage of the acetone powder extract over fresh extract is a low content in adenosine-triphosphatase of the former. For preparation of the acetone liver powder, pigeons are killed by decapitation and bled as completely as possible. The livers are quickly removed and chilled until used. The livers are weighed and a volume of chilled acetone, 20 times the weight in gm. of the livers, is placed in a chilled Waring mixer. The livers are now minced, transferred to the mixer, and homogenized with the acetone for 2 minutes. The homogenate is rapidly filtered on a Büchner funnel and the residue is washed on the funnel with acetone and ether, with care not to pack too tightly. The ether should be checked for peroxide, the presence of which destroys the enzyme preparation. The homogenization is carried out in the cold room. When well chilled acetone and ether were used, it was not found necessary to carry out the filtration under refrigeration. The powder is eventually dried in a vacuum desiccator over phosphorus pentoxide. Connective

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tissue is removed by passing the powder through a sieve. From 15 to 20 gm. of pinkish powder are obtained from 100 gm. of fresh liver, corresponding to ten to twelve pigeons.

We have observed that the pink color is a helpful index for the activity of the preparation. The presence of undenatured hemoglobin gives this reddish tinge. If, however, most of the hemoglobin is denatured, a brownish acetone powder results which invariably has poor activity. We find that at least 15, and even better 20, volumes of acetone are necessary to give satisfactory preparations, and that the purity of the ether must also be controlled with great care.

#### *Preparation and Aging of Extracts*

The extract is prepared by carefully rubbing up 10 gm. of liver powder with 100 ml. of 0.02 M sodium bicarbonate solution. The insoluble residue is removed by centrifugation. Between 60 and 75 ml. of dark reddish, slightly opalescent fluid are obtained. This has now to be kept frozen overnight in a deep freeze chamber. For inactivation, the material is thawed, brought to room temperature, and allowed to stand for 4 hours. This produces 95 to 100 per cent inactivation. After aging, the extract is recentrifuged, and the insoluble material discarded. The aged extract can be kept indefinitely by deep freezing.

It is important to freeze the extracts before aging, as freezing precipitates a large amount of adenosinetriphosphatase. Recentrifugation should be carried out *after* aging or else the preparation loses much of its activity. For example, a pigeon liver extract, which was centrifuged first and then aged, acetylated 16.0  $\gamma$  of sulfanilamide, while a comparable extract, when first aged and then centrifuged, acetylated 44.2  $\gamma$  of sulfanilamide per ml. The aged recentrifuged extract should be a reddish, water-clear solution.

The rate of breakdown of coenzyme A in the extract is shown in Table I. In the earlier experiments, inactivation was carried out by allowing the extract to stand overnight at 5–8° (2). Table I shows that a more complete inactivation is obtained by autolysis for 4 hours at room temperature. The enzyme mainly responsible for the inactivation is unstable and its activity decreases during incubation at room temperature to a small fraction of the original. This circumstance is of advantage for the use of the extract in the assay of coenzyme A.

#### *Assay Procedure*

If aged extract is supplied with sulfanilamide, acetate, and adenosine triphosphate and cysteine and citrate are added as stabilizers, the rate of acetylation becomes exclusively a function of the concentration of coenzyme A. Cysteine reactivates and stabilizes the acetylation enzyme. In our

experience, citrate exerts its influence largely, if not exclusively, by suppressing residual adenosinetriphosphatase activity through the deionization of calcium; in further purified preparations, the stimulatory effect of citrate disappears together with adenosinetriphosphatase activity.

For the routine assay of coenzyme A, it is convenient to combine most of the reagents into the following reaction mixture: 10 ml. of 0.004 M sulfanilamide, 2.5 ml. of M sodium acetate, 8.0 ml. of approximately 0.05 M potassium adenosine triphosphate, 10 ml. of 0.2 M sodium citrate.

The pH of the mixture should be around 7. Larger samples are divided into small portions of 4 to 5 ml., which are kept frozen until used. Tubes of 1 to 1.3 cm. in diameter are used for the assay. It is advisable not to increase unduly the proportion of surface to volume in order to reduce the

TABLE I  
*Breakdown of Coenzyme in Liver Extract*

Incubation time <i>hrs.</i>	Per cent decomposition	
	25°	4°
1	55	
2	78	24
3	95	
4	100	41
8		76
16		80
24		85

oxidation of cysteine during the experiment. Under the conditions described above, no difference was found when air was replaced by nitrogen.

To each tube there are added (1) the solution to be tested for coenzyme activity (this volume should not exceed 0.3 ml.), (2) 0.3 ml. of the assay reaction mixture, (3) 0.08 ml. of a freshly prepared M sodium bicarbonate solution, (4) 0.1 ml. of 0.1 M cysteine hydrochloride, (5) 0.25 ml. of the aged enzyme, (6) water to volume. A blank is prepared by adding all the constituents except coenzyme. The tubes are stoppered, mixed, and incubated for 2 hours in a water bath of 37°. The reaction is halted by adding 4 ml. of 5 per cent trichloroacetic acid. After centrifugation, aliquots of 1 ml. are taken for sulfanilamide determinations, carried out by the procedure of Bratton and Marshall (3). Readings were made on a Klett photoelectric colorimeter, with Filter 54. The difference in sulfanilamide between the blank and the tubes containing coenzyme represents the amount of sulfanilamide acetylated.

*Concentration-Activity Curve and Coenzyme A Unit*

In Fig. 1 there is presented the response of aged enzyme to increased amounts of coenzyme A. With the same enzyme preparation data are compared for crude, boiled liver extract and for highly purified coenzyme. The concentration-activity curves for these two preparations of coenzyme overlap almost exactly, which shows an independence of relative activity of the degree of purity. At medium concentrations, the curve is nearly a straight line, and it appeared logical to choose the point of half reaction as the unit. *Such an amount, therefore, as will activate our system to half the maximum activity is defined as 1 unit of coenzyme A.* The unit is largely independent of the individual enzyme preparation, even if the absolute amounts of acetylated sulfanilamide may vary. The coenzyme A unit contains 0.7  $\gamma$  of bound pantothenic acid and is independent of the purity of the preparation (1).

It may be observed that, after reaching near saturation, the curve still tends to climb very slowly. Such small increases are disregarded in our definition of the unit of coenzyme A, a practical saturation point being fixed at 3 to 4 units.

At coenzyme A saturation, a good enzyme preparation will acetylate 75 to 85 per cent of the added sulfanilamide. Best readings are therefore obtained in the region of the curve where approximately 40 per cent of the sulfanilamide disappears during incubation. Extracts which show considerably less acetylation should be discarded. In some cases, a weak extract may be improved by addition of an extra 0.1 ml. of 0.1 M cysteine.

*Standard Coenzyme*

A potent coenzyme solution is prepared by quickly boiling fresh minced pigeon, rat, or rabbit liver with 3 volumes of water. To make a dry preparation of coenzyme A, this solution is brought to pH 2 with hydrochloric acid and then is precipitated with 10 volumes of acetone; the precipitate is washed with acetone and ether and dried. This preparation is hygroscopic and deteriorates on wetting. When kept in a desiccator, however, in a cool place, it is very stable. Such crude coenzyme A preparations contain 0.25 to 0.5 unit per mg. of dry matter. This preparation is used as the reference coenzyme. With it, an activity-concentration curve (Fig. 1) is prepared, and the amount corresponding to the unit is noted.

For evaluation of unknown samples, we test at two levels, slightly below and above the unit point respectively, and take the average. In each assay run are included the unit and the saturation points with the reference coenzyme. The reading for the test sample, if not too far removed from the unit point, is divided by the reading for the unit of the reference coenzyme.

This yields directly the number of units contained in the unknown. A somewhat more accurate, although a more laborious, method of standardization is to prepare a curve with reference coenzyme for every batch of extract over a wider range of concentrations. From this standard curve, the coenzyme content of the test sample may be read directly. A new curve has to be prepared for every new extract, even if it is made from the same batch of acetone powder. In deep freezing, however, the extract does not change.

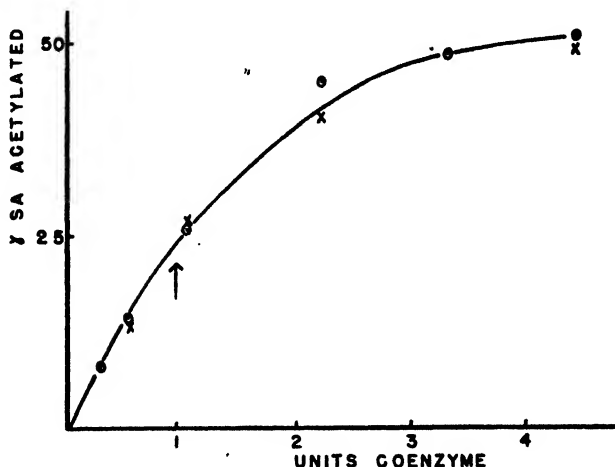


FIG. 1. Concentration-activity curves for coenzyme A preparations of different purity. The arrow indicates the point of 1 unit on the curve. Ordinate scale, sulfanilamide acetylated. O, crude coenzyme, 0.25 unit per mg.; X, purified coenzyme, 130 units per mg.

#### *Coenzyme A Content of Animal Tissues, Microorganisms, and Plant Materials*

Preliminary data on the distribution of coenzyme A were published previously (2, 4). Some further data on the distribution of the activator of choline acetylation in brain, which is identical with coenzyme A (5), were reported by Feldberg and Mann (6) and Nachmansohn and Berman (7).

For the assay of coenzyme A in tissues, animals are killed by decapitation and the organs quickly removed and chilled. The organs are minced or homogenized with the appropriate volumes of water and rapidly boiled. The boiled extracts are then centrifuged or filtered and assayed for the coenzyme.

In this procedure, little or no coenzyme is lost by autolysis. This was checked by microbiological determination of free and coenzyme A-bound<sup>1</sup>

<sup>1</sup> Novelli, G. D., Kaplan, N. O., and Lipmann, F., in preparation.

pantothenic acid. Little or no free pantothenic acid was found in fresh tissues. Therefore, most or all of the pantothenic acid is bound in coenzyme A. Detailed data on this point will be reported subsequently.

Table II shows that considerable amounts of coenzyme A are present in all parenchymatous organs. The highest amounts are found in liver, as appears to be the general rule for all coenzymes. Liver seems to act to some extent as a storage organ. It was observed that the content of coenzyme A in liver fluctuates more with variation of pantothenic acid intake than does the content of other organs.

It is noteworthy that the organ nearest to the liver in coenzyme A con-

TABLE II

*Coenzyme A in Animal Tissues*

All values are given in units of coenzyme A per gm. of fresh tissue.

	Human	Rabbit	Rat	Pigeon
Liver.....		112	132	105
Adrenal.....		65	91	
" demedullated*....			79	
Kidney.....		50	74	
Brain.....		40 (Cortex)	28	40
Heart.....		26	42	45
Testes.....		26		
Intestine.....			26	
Thymus.....			20	
Skeletal muscle.....		6		
Blood plasma.....	0			
Red blood cells.....	3-4			

\* We wish to thank Dr. H. W. Deane and Dr. R. O. Greep for the demedullated glands.

tent is the adrenal gland. Most of the coenzyme A must be present in the cortex, since demedullated adrenals show essentially the same amounts as do the whole adrenals. It is tempting to see a relationship between this high content in coenzyme A and the adrenal cortex degeneration in pantothenic acid deficiency (8). A possible rôle of coenzyme A in the manufacture of cortical hormone may be suggested by its function in acetate metabolism (9, 10). Acetate has been reported as being a building stone of substances of steroid structure (11).

In Table II data on human blood are included. Coenzyme A was found absent in plasma, but red blood cells contained relatively large amounts, 3 to 4 units per ml., which corresponds to approximately 2  $\gamma$  of pantothenic acid. Values of 0.2 to 0.4  $\gamma$  per ml. (12), reported for pantothenic acid in

blood cells, therefore appear too low, owing probably to an incomplete recovery of pantothenic acid from coenzyme A.

Among the data on coenzyme A content in microorganisms, there is included a considerable number of values which were obtained with dry preparations, some of which had been stored for some time. These preparations were obtained by drying the organisms in a thin layer over phos-

TABLE III  
*Coenzyme A in Microorganisms\**

Microorganism	Coenzyme A per gm. dry weight	Preparation used for assay
	<i>units</i>	
<i>Proteus morganii</i> .....	572	Freshly grown organism, boiled
<i>Lactobacillus arabinosus</i> .....	150	
" <i>delbrueckii</i> .....	40	Dry preparation, sus- pended in water and boiled
Tray-dried yeast.....	72	
" ".....	41	
<i>Escherichia coli</i> .....	320	
Propionic acid bacteria.....	330	
<i>Clostridium butylicum</i> (dried extract)....	2000	

\* We wish to thank Mr. G. D. Novelli for collaboration in these experiments.

TABLE IV  
*Coenzyme A in Plant Material*

	Coenzyme A per gm. fresh weight
	<i>units</i>
Spinach.....	0.74
Tomato.....	1.3
Frozen peas.....	4.5
Wheat germ (commercial sample).....	30
Royal jelly (bee)*.....	0

\* Kindly supplied by Dr. Thomas S. Gardner.

phorus pentoxide, during which some autolysis may have occurred. The data obtained with these materials are to be considered minimum values. The figures in Table III show a considerable variation in coenzyme content in various microorganisms. The highest content so far observed in nature, 2000 units per gm. of dry matter, was found in *Clostridium butylicum* extract prepared according to the method of Koepsell and Johnson (13). This observation, together with the observed rôle of coenzyme A in acetate metabolism (9), seems to suggest a function of coenzyme A in the condensation of 2 carbon residues to 4 carbon chains.



Comparison of the data for coenzyme A in microorganisms and tissues shows that the coenzyme appears to be present in similar concentrations in both groups. It is to be observed that the data for animal tissues are given for fresh weight and are to be multiplied by a factor of 4 for such comparisons. The content in plant material is generally lower. Peas, however, contain considerable amounts, and a high content was found in a commercial wheat germ preparation. The royal jelly of the bee was tested because of its unusually high content of pantothenic acid (14) but no coenzyme A was found. See Table IV.

In general, the ubiquitous presence of coenzyme A in living cells soon suggested a more important function for it than merely that in the acetylation system in which its activity was first observed. Recent observations tend to amplify this early suggestion (9, 10).

#### SUMMARY

A method is described for the assay of the pantothenic acid derivative, coenzyme A. Pigeon liver extract which undergoes autolysis loses its ability to acetylate sulfanilamide. The acetylating activity is recovered by addition of coenzyme A. At medium concentration, reactivation is proportional to coenzyme A concentration.

A unit of coenzyme A is defined as that amount which reactivates our system to half the maximum activity.

Data are presented on the coenzyme A content of animal tissues, microorganisms, and plants. It was found to be a general constituent of living organisms. The highest values were observed with liver, *Clostridium butylicum*, and *Proteus morganii*.

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# A METHOD FOR COMPUTING THE EFFECTIVE EXTRANEOUS REDUCERS IN THE FOLIN SPECTROPHOTOMETRIC DETERMINATION OF CYSTINE\*

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The basic Folin method (1) for the determination of cysteine and cystine is dependent upon the blue color resulting from the reduction of phosphotungstic acid by the sulfhydryl group of cysteine. For the determination of cystine, one-half of its molecule is converted to cysteine by cleavage with sulfite (2). Under the conditions of sulfite treatment, the color due to cysteine itself is doubled. The color reaction is complicated, however, by the fact that the phosphotungstic acid reagent is reduced not only by cysteine but also to a certain extent by so called "extraneous reducers." Lugg found (3) that the action of reducers other than cysteine may be increased by sulfite and proposed mercaptide formation of cysteine with mercuric chloride as a means of isolating the non-cysteine color, only to discover that the reducing effect of the extraneous reducers is enhanced when cysteine is thus eliminated. He overcame this difficulty by adding to the mercuric chloride blank a known amount of ferrous ion in quantity approximately equivalent to the amount of cystine present. Ferrous ion reduces phosphotungstic acid quantitatively, and by subtracting its contribution from the total color Lugg obtained the color value of the extraneous reducers as it appears in the presence of the large amount of reduced phosphotungstate. Kassell and Brand (4), in developing a modification of the Lugg method for use with the Pulfrich photometer, also recognized the fact that the direct mercuric chloride blank is too large. They replaced the ferrous ion method of Lugg by a scheme in which two mercuric chloride blanks were used, one with and one without sulfite. By averaging the resulting two values, they empirically arrived at what they considered to be approximately correct results.

In connection with an investigation of the sulfur balance of a number of proteins, the Kassell and Brand method has been adapted for use with the Coleman model 11 spectrophotometer. With their method for computing the blank value, inconsistencies were encountered in results obtained on

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HCl-HCOOH hydrolysates of proteins, and generally on proteins containing substantial amounts of nucleic acid. These difficulties manifested themselves in cystine values varying with the amount of hydrolysate taken for analysis. It became necessary, therefore, to evaluate the blank values more critically by comparing them with those obtained by the ferrous ion method, which appears to be the most reliable if not the most convenient method of correction.

The results have revealed certain systematic relations between total reducers, apparent extraneous reducers, and true extraneous reducers which can be described by a nomograph. It appears that, within the range of materials studied, application of the nomograph permits a simpler evaluation of the blank than was heretofore possible. In addition, data have been obtained pertaining to the relation between method of hydrolysis and maximum cystine values.

#### EXPERIMENTAL

*Proteins Used*—Commercial egg white (egg albumin, Merck, impalpable powder, soluble), bovine plasma albumin (Armour Laboratories, Chicago), Tissue Protein TP3SM (a representative mixture of all the protein fractions obtained in this laboratory from a human lymphosarcoma), edestin (Hoffmann-La Roche), lactalbumin (Labco 7-HAAX),<sup>1</sup> reduced lactalbumin (lactalbumin reduced by thioglycolate),<sup>1</sup> and casein (Labco).<sup>1</sup>

*Hydrolysis*—At the outset of the investigation, a series of hydrolysis experiments was run in order to establish optimum conditions for obtaining reproducible and constant values for cystine in proteins. Dried egg white was used as the trial protein. Hydrolyses were carried out in a sealed tube in a pressure cooker kept at 120° in a constant temperature oven. The hydrolytic media used were 3 N HCl, 3 N HCl *in vacuo*, and a 1:1 mixture of 98 per cent formic acid and 6 N HCl *in vacuo* (a modification of the method of Miller and du Vigneaud (6)). Samples consisted of approximately 200 mg. of protein and 2 cc. of acid.

As shown in Fig. 1, the HCl-HCOOH medium yielded the best results. Other proteins hydrolyzed by this method behaved similarly. A protein fraction derived from a tumor nucleoprotein preparation yielded 0.85, 0.85, and 0.82 per cent cystine at 8, 16, and 24 hours, respectively, and bovine plasma albumin gave identical values of 6.02 per cent cystine at 6 and 16 hours. The method, therefore, permits of wide latitude in the time of hydrolysis.<sup>2</sup> Kassell and Brand (7) did not recommend HCl-HCOOH as

<sup>1</sup> We are indebted to Dr. E. Brand for the samples of casein, lactalbumin, and reduced lactalbumin used in these analyses. Their description, method of preparation, and cystine and cysteine content were reported by Kassell, Cahill, and Brand (5).

<sup>2</sup> Similar experiments on methionine recovery showed that the HCl-HCOOH method, while satisfactory for hydrolyzing egg white, did not prevent losses of

a hydrolytic medium because they considered it necessary to remove the formic acid before analysis. We have found that under our conditions the analysis is not affected by the presence of formic acid and, in addition, that its removal by evaporation of the hydrolysate resulted in decreased cystine values (4 per cent and 7 per cent in two cases). Therefore, the analyses were, as a rule, carried out without removal of formic acid. In one series of determinations on the tissue protein (Sample 5EN, Table II), the hydrolysate was evaporated in a desiccator in order to study the effect of the removal of volatile substances on the blank value. In order to obtain a variety of

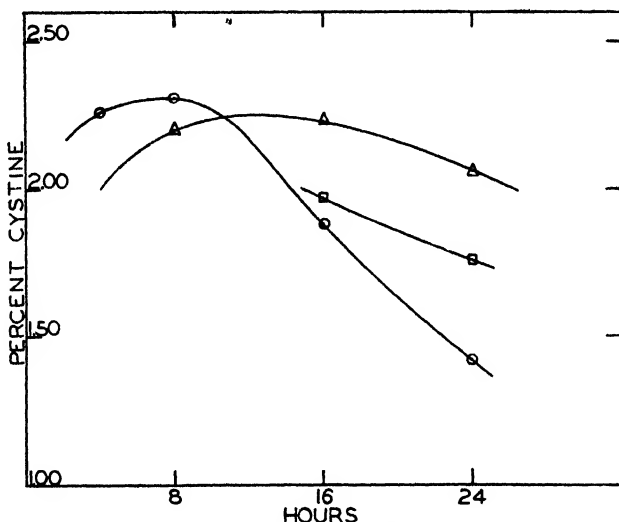


FIG. 1. Rate of cystine loss in egg white in different hydrolytic media. O, 3 N HCl, 120°; □, 3 N HCl, 120°, *in vacuo*; Δ, 3 N HCl-50 per cent HCOOH, 120°, *in vacuo*. The slightly lower maximum value for this last curve is due to the removal of HCOOH by evaporation, which was later found to cause small losses in cystine content.

blank conditions, a series of determinations was also included on a hydrochloric acid hydrolysate of egg white.

**Decolorization**—While the use of a spectrophotometer ordinarily makes the removal of soluble humins unnecessary, it was found that at the pH of the analysis precipitating humins often caused turbidities. Unless otherwise noted, the hydrolysates were therefore adjusted to pH 4.5 to 4.8 and, after 20 minutes, filtered. A later check on this method showed that under these conditions any cysteine present undergoes rapid oxidation to cystine, so that only the sum of cystine and cysteine is being measured.

methionine after 8 hours of hydrolysis in the case of a protein fraction derived from a tissue nucleoprotein. It is evident that the conditions of hydrolysis must be carefully investigated for the particular amino acid and protein being analysed.

In the case of casein, the lactalbumins, and edestin, listed in Table II, the hydrolysates were not decolorized or neutralized previous to color development and any resulting turbidities were removed by filtration through a sintered glass filter.<sup>3</sup>

*Analytical Method.*—The reagents and general procedure are those of Kassell and Brand (4). The various reaction mixtures used are summarized in Table I. The final volume in each case was 25 cc. Readings were made in 1.31 cm. square cuvettes at a mean wave-length of 720 m $\mu$ . Spectrophotometric values are reported in optical density units equivalent

TABLE I  
*Composition of Reaction Mixtures*

	Kassell and Brand					Fe <sup>++</sup> method (Lugg)*		
	Solu- tion A	Solu- tion B	Solu- tion D	Solu- tion E'	Solu- tion F	Solu- tion G	Solu- tion H	Solu- tion I
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Buffer, pH 5.7 . . . . .	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
HgCl <sub>2</sub> , 0.25 M . . . . .	0	0.1	0.1	0	0.1	0.1	0.1	0.1
H <sub>2</sub> O . . . . .	To make reaction volume of 8 cc.							
Hydrolysate . . . . .	1	1	1	0	1	0	1	0
NaOH . . . . .	†	†	†	0	†	0	†	0
Phosphotungstic acid . . . . .	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Buffered sulfite . . . . .	0.25	0.25	0	0	0.25	0	0.25	0
Fe(NH <sub>4</sub> ) <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub> . . . . .	0	0	0	0	‡	‡	§	§

\* The use of two levels of ferrous ion concentration in the blanks, followed by interpolation between them, is our refinement of the original Lugg method.

† Sufficient NaOH to neutralize the acidity of the aliquot of the hydrolysate, as determined by previous titration to the methyl red end-point.

‡ To yield an amount of color approximately half that of solution A.

§ To yield an amount of color approximately equal to that of solution A.

to  $1000 \times \log I_0/I$ , where  $I_0$  is the intensity of the incident light and  $I$  that of the transmitted light. The photometer blank consists of buffer plus phosphotungstic acid (solution E'). It is our experience that the phosphotungstic acid reagent contributes an appreciable amount of light absorption to the solution tested, corresponding to about 10 optical density units. While in the actual determination this correction cancels out by subtraction of the blank, it is important in the preparation of the calibration curve. Unless readings are made against a phosphotungstic acid photometer blank, the ratio of color to cystine concentration will markedly increase at low concentrations because of the contribution of the reagent

<sup>3</sup> It is our experience that filter paper selectively adsorbs the blue reduced phosphotungstic acid.

color; *i.e.*, one does not obtain a constant molar extinction coefficient. The molar extinction coefficient for cysteine-reduced phosphotungstic acid at 720  $m\mu$  was found to be  $6910 \pm 20$  over a range of  $5.05 \times 10^{-4}$  to  $26.0 \times 10^{-4}$  mm of cystine per 25 cc. of final solution.

In solution A there is developed the total color of cystine and of the

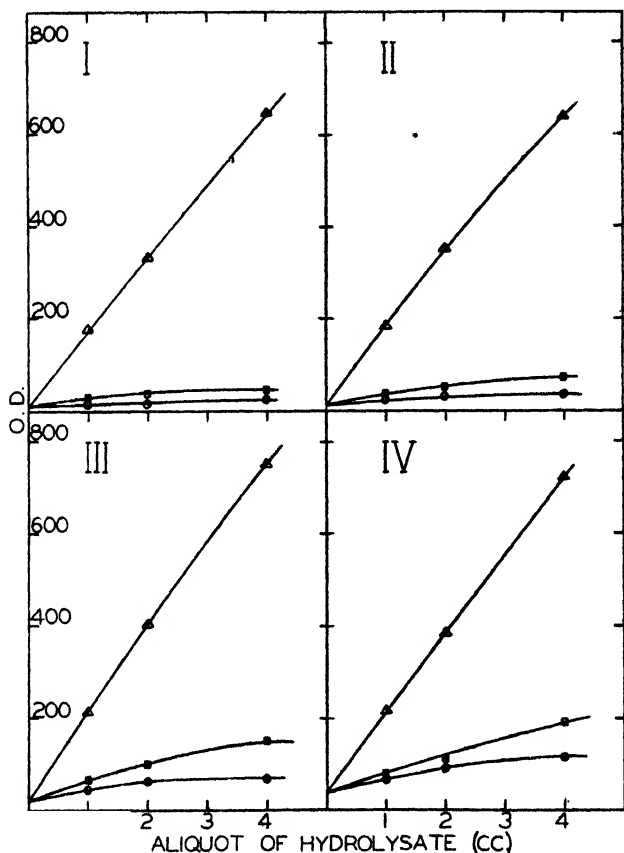


FIG. 2. Optical densities of the total color A ( $\Delta$ ), observed blank B ( $\square$ ), and blank B' corrected by the ferrous method of Lugg ( $\circ$ ). I, bovine plasma albumin; II, egg white; III, Tissue Protein TP3SM, Sample 5EN, IV; Tissue Protein TP3SM, Sample 4N (see foot-note to Table II).

extraneous reducers; solution B gives the extraneous reducers with sulfite, and solution D shows the color caused by the extraneous reducers without sulfite. By averaging the two blanks, B and D, Kassell and Brand obtain their correction for extraneous reducers. In solutions F and H, cysteine, inactivated by mercuric chloride, is replaced by ferrous ion, in accordance

with Lugg's procedure. The amounts of ferrous ion used in solutions F and H correspond respectively to approximately one-half and all of the color of solution A. At each level a concurrent determination on the same amount of ferrous ion solution without any hydrolysate is run (solutions G and I). The differences between F and G and between H and I represent

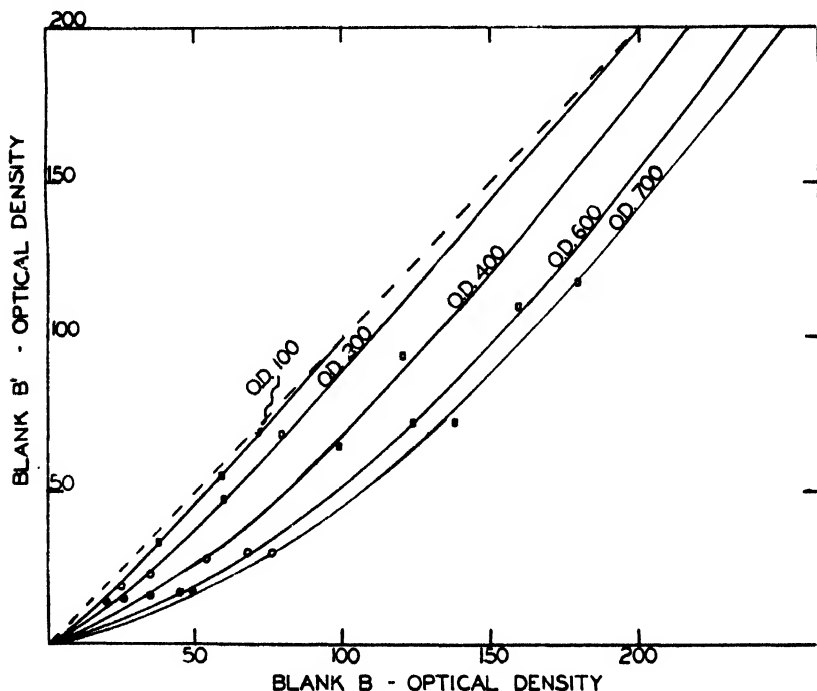


FIG. 3. Relation of blank B' ( $\text{HgCl}_2$ ,  $\text{SO}_3^{2-}$ , and  $\text{Fe}^{++}$ ) to blank B ( $\text{HgCl}_2$ ,  $\text{SO}_3^{2-}$ ) at different levels of total optical density. ●, bovine plasma albumin; ○, egg white; ■, Tissue Protein TP3SM, Sample 5EN; □, Tissue Protein TP3SM, Sample 4N. The broken line is the locus of the optical densities of all solutions in which cystine and cysteine are absent and in which B' (corrected blank) is equal to the determined blank, solution B, and both are equal to the color of solution A.

the colors corresponding to the extraneous reducers at the total optical densities of solutions F and H, respectively. From the resulting blanks at these two levels, it is a matter of interpolation to calculate the optical density of the extraneous reducers in the presence of the color obtained in solution A of the determination. This corrected blank is referred to as B' hereafter. The difference between the color values A and B' is the optical density due to the total cystine present.

### Results

The first proteins studied were egg white, bovine plasma albumin, and two tissue protein samples. Analyses were run on 1, 2, and 4 cc. aliquots of the hydrolysates. The resulting optical densities corresponding to A, B, and B' are shown in Fig. 2. It is notable in these graphs that in each case the values of A, B, and B' coincide, within the limits of error, at zero amount of hydrolysate, indicating that the color contributed by the sulfite

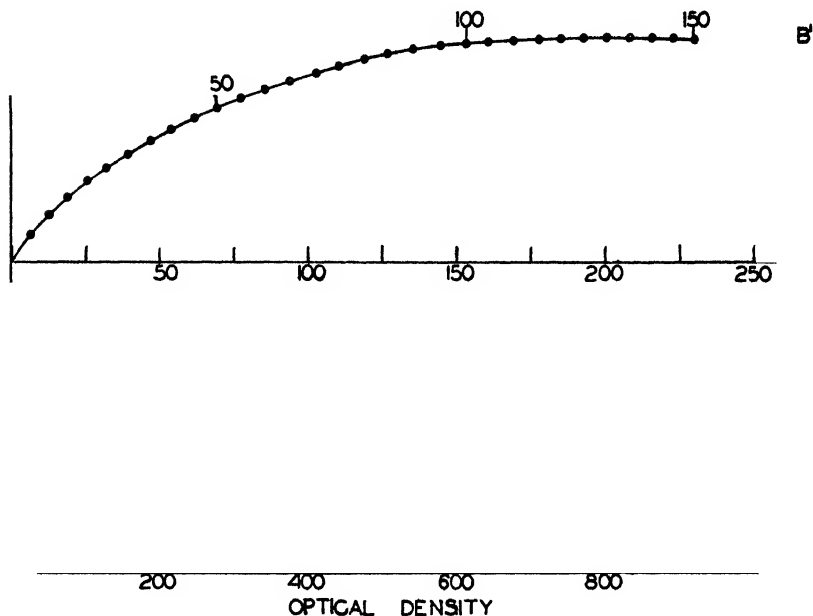


FIG. 4. Nomograph for determining the adjusted blank value B' from the determined values of total color (solution A) and mercuric chloride blank (solution B)

is independent of the added mercuric chloride and therefore cancels out in the final calculation. However, the graph also shows that, for reasons not apparent, the magnitude of this sulfite color differs for different hydrolysates.

The decreasing slope of the corrected B' curves with increase of total color in solution A suggests a suppressing action of the reduced phosphotungstate on the reaction of the extraneous reducers. This tendency, evident in all the proteins tested, presented the possibility that a general relationship exists between the corrected blank values (B') and the de-



TABLE II  
Cystine Values Obtained by Three Methods for Computing Blank

Protein*	Hydrolytic treatment†	Aliquots analyzed	Cystine content found (range and average) according to		
			Lugg (Fe)	Nomograph	Kassell and Brand
		cc.	per cent	per cent	per cent
Bovine plasma albumin	(a)	1, 2, 4	5.80-5.94 5.85	5.80-5.89 5.85	5.50-5.72 5.63
Egg white N	(b)	1, 2, 4	2.26-2.43 2.36	2.28-2.43 2.35	2.21-2.36 2.28
Tissue Protein TP3SM, Sample 5EN	(a)	1, 2, 4	1.25-1.30 1.28	1.22-1.28 1.26	1.24-1.25 1.25
Tissue Protein TP3SM, Sample 4N	"	1, 2, 4	1.34-1.43 1.40	1.40-1.46 1.43	1.41-1.55 1.47
Tissue Protein TP3SM, Sample 1N	"	1, 2, 3, 4		1.38-1.49 1.45	1.37-1.60 1.48
Tissue Protein TP3SM, Sample 2EN	"	3, 4		1.36-1.36 1.36	1.31-1.33 1.32
Tissue Protein TP3SM, Sample 3EN	"	1, 2, 3, 4		1.27-1.39 1.34	1.30-1.38 1.33
Tissue Protein TP3SM, Sample 4EN	"	1, 2, 4		1.36-1.38 1.38	1.35-1.38 1.37
Casein N	(c)		0.321	0.328	0.328
Lactalbumin	(a), 8 hrs.		3.00	2.99	2.99
	" 16 "		2.94	2.92	2.89
Reduced lactalbumin‡	" 8 "		2.55	2.57	2.58
	" 16 "		2.54	2.62	2.54
Edestin	" 8 "		1.18	1.18	1.16
	" 16 "		1.21	1.20	1.18

\* The symbol E indicates removal of the formic acid after hydrolysis by evaporation, while N indicates neutralization of the hydrolysate (pH 4.5), followed by filtration of the precipitated humins before analysis.

† (a), 3 N HCl-50 per cent HCOOH, sealed tube *in vacuo*, 120°, 8 hours and, for the last three proteins, also 16 hours; (b), concentrated HCl, steam bath for 18 hours, boiled under a reflux for 8 hours; (c), 3 N HCl, sealed tube *in vacuo*, 120° for 8 hours.

‡ The cysteine content of the reduced lactalbumin was found to be 0.18 and 0.13 per cent after 8 and 16 hours of hydrolysis, respectively. Dr. E. Brand's own determination gave 0.53 per cent cystine and 2.5 per cent cysteine. The cysteine determinations were repeated twice, in these cases hydrolyzing for 8 hours and analyzing within 30 minutes, without significant change in the results. We conclude that the thioglycolate-reduced lactalbumin has undergone extensive reoxidation during storage (about 10 years).

terminated A and B values. To explore this possibility, the data obtained with the different protein hydrolysates were combined into one graph by selecting the total optical density levels (determination of solution A) of 100, 200, 400, 600, and 700 and for each of these levels correlating all values of solution B with the corresponding B' values. This resulted in the graph shown in Fig. 3. The similarly designated points represent each of the proteins and the curves connecting the points correspond to constant total optical density levels of solution A.

The plotted data from these four hydrolysates seem to confirm a functional relationship. Besides accounting for the experimental points, they also adequately cover the limiting conditions of (a) a cystine-free hydrolysate and (b) a pure cystine solution. In both of these cases the B and B' blanks are equal and in case (b) amount to zero. Fig. 4 shows a nomograph which is constructed from the ideal values of Fig. 3. Within the ranges covered, Fig. 4 permits direct reading of the B' value corresponding to any combination of experimentally found values for solutions A and B by projecting a line connecting the points corresponding to solutions A and B on the two horizontal lines to the curved scale of B' values.

With the aid of this nomograph, results obtained on a number of hydrolysates in addition to the ones constituting the basis for the nomograph were calculated. In Table II are shown the values obtained on each hydrolysate by the method of Kassell and Brand or by the nomograph method, and in most cases also those obtained by the Lugg ferrous procedure.

On the whole, it seems that there is little difference in the results obtained by the various methods for calculating the blank. However, some variation may be noted in the case of hydrolysates with high blank values, such as the tissue protein, Samples 4N and 1N, and also in those with very low blanks, such as the bovine plasma albumin. The former proteins yield high results, while the latter tend to be low with the Kassell and Brand method, as compared with that of Lugg. It is only by using the higher values obtained with the nomograph on bovine plasma albumin that all of the sulfur could be accounted for as cystine and methionine.

#### DISCUSSION

As has been demonstrated by others, reduced phosphotungstic acid has a pronounced effect on the development of color by extraneous reducers. The mechanism may be either a shift in the equilibrium or a slowing of the rate of reaction of these reducers with phosphotungstic acid. In either case, inhibition is definite and the application of the mercuric chloride blank alone fails to yield correct results. This inhibition is a function of both the total color produced by the cysteine and cystine and the amount of extraneous reducers present. The systematic relationship between these

variables, as exhibited by Figs. 3 and 4, seems to be practically independent of the nature of the protein hydrolysate. Because of this relationship a convenient nomographic procedure may be substituted for the reliable and theoretically well founded Lugg method. While the empirical corrections of Kassell and Brand yield, in general, results as good as those obtained with the Lugg method, they fail when they are applied in the extreme conditions of very high blanks, such as was shown in the tissue protein, Samples 4N and 1N, and those with very low blanks, as in the case of bovine plasma albumin. Under these conditions, the spread of values obtained by analyzing varying amounts of hydrolysate is decreased by applying either the Lugg or the nomograph method.

#### SUMMARY

1. The Folin method for the determination of cystine, as modified by Kassell and Brand, has been investigated with the aim of obtaining a more reliable estimation of the correction due to extraneous reducers.

2. Methods of hydrolysis are described which yield nearly constant maximum values for total cystine in proteins.

3. In HCl-HCOOH hydrolysates, evaporation of the hydrolysate in order to remove formic acid causes loss of cystine. The presence of formic acid, on the other hand, does not affect the analytical method itself.

4. When the absorption is measured with the Coleman spectrophotometer at a wave-length of 720  $m\mu$ , the phosphotungstic acid present in the solution increases the amount of absorption appreciably. Phosphotungstic acid, therefore, must be included in the photometer blank in preparing a calibration curve. The molar extinction coefficient for cysteine-reduced phosphotungstic acid was found to be  $6910 \pm 20$  at this wave-length.

5. The degree to which extraneous reducers react with phosphotungstic acid is a positive function of their concentration and a negative function of the total concentration of reduced phosphotungstic acid. This relation appears to be independent of the specific nature of different types of protein hydrolysates and can be adequately described by a nomograph in which total color and color obtained when cysteine is inactivated by mercuric chloride are the only variables.

6. The relationship described in terms of the nomograph gives as good a basis for the estimation of the corrected blank as Lugg's ferrous ion substitution method or Kassell and Brand's scheme of using the mean value of the mercuric chloride blanks obtained in the presence and absence of sulfite.

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# COUNTER-CURRENT DISTRIBUTION STUDIES ON STREPTOMYCIN: THE TAUTOMERISM OF STREPTOMYCIN

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The simplicity and reproducibility which characterize the conversion of streptomycin to maltol (1) by heating in dilute alkali have made this reaction the basis of a frequently used analytical method for streptomycin (2). When used in these laboratories as a method of assaying streptomycin preparations, however, the maltol reaction consistently gave lower yields per unit of potency with pure streptomycin trihydrochloride or its calcium chloride double salt than with impure preparations of lower activity. This observation suggested the possibility that the cruder concentrates contained other maltol precursors less active than streptomycin, a supposition which has been confirmed by the isolation of mannosidostreptomycin<sup>1</sup> (5, 6).

In a preliminary communication (7) it was remarked that the broad distribution curves obtained by the application of the Craig counter-current distribution technique (8, 9) to solid streptomycin preparations assaying approximately 400 units per mg. could be considered to indicate the presence of at least three maltol-producing entities (*cf.* Curve B (7)). The distribution studies herein described were originally undertaken as part of the effort to isolate the hypothetical third streptomycin-like substance, but they have now shown that this third fraction does not represent such an entity and have instead yielded evidence that streptomycin exists in several tautomeric modifications, the relative proportions of which depend on the pH. These tautomers differ in the ratio of distribution between some immiscible solvent pairs and are at least partially separable by adsorption on alumina.

In order to concentrate the different components, the crude mixture of streptomycin (400 to 500 units per mg.) was fractionated by liquid chroma-

<sup>1</sup> It has been suggested by Dr. Selman A. Waksman (*Science*, **107**, 233 (1948)) that the term streptomycin be reserved for the pure substance  $C_{21}H_{39}N_7O_{12}$  described by Peck *et al.* (3) and by Fried and Wintersteiner (4). The Squibb investigators (Fried, Stavelly, Titus, and Wintersteiner) have cooperated with Dr. Waksman by substituting the term mannosidostreptomycin for streptomycin B. The new name is based on data to be published shortly by Dr. Stavelly and Dr. Fried. Crude concentrates containing unknown proportions of the two streptomycins would be referred to under the revised nomenclature as "streptomycin complex."

tography with dry methanol and acid-washed alumina (10, 11). The solid residues obtained by lyophilization of each chromatographic fraction were then subjected to counter-current distribution in butanol and 5 per cent *p*-toluenesulfonic acid (7). Since for each constituent of a mixture examined by the latter technique the distribution curve will show a maximum, the location of which is a simple function of the partition coefficient (12), it proved convenient to refer to the various fractions obtained in terms of their distribution constants between the phases used. These constants, which may be readily calculated from the curves (12), represent the ratio of concentration in butanol over concentration in the aqueous phase, and are for the sake of brevity hereinafter referred to as *K*. The twenty-four plate distribution curves illustrated in this paper are all plotted so that fractions appear from left to right in the order of increasing distribution constants, substances with a *K* of 1 having their maximum concentration in the center.

Distribution quickly revealed that chromatography had effected the separation of three types of material. That obtained in the first three or four fractions from the column contained 20 to 40 per cent of what appeared from the broad distribution curves to be an ill defined mixture of maltol-producing substances with *K* ranging from 1 to 10. Streptomycin, with a *K* of unity, accounted for the remainder of the first fractions and occurred practically pure in subsequent ones. This yielded place to mannosido-streptomycin (5, 6) as the more strongly adsorbed fractions emerged from the column, a process which was reflected in the distribution curves by the disappearance of the central band corresponding to a *K* of 1 and the appearance of a peak corresponding to a *K* of about 0.4. This paper will be concerned only with the early chromatographic fractions in which mannosidostreptomycin had not yet appeared.

Curve A in Fig. 1 illustrates the distribution in a 5 per cent *p*-toluenesulfonic acid system of a typical early chromatographic fraction. The broad curve obtained is open to two interpretations, the immediately obvious one being the presence of several substances with *K* ranging between 1 and 10. Such curves would also result, however, if material moving far to the right of the distribution pattern because of its high *K* was to be gradually converted during the course of the distribution to a substance of lower partition coefficient. In that event, part of the material would be left behind as the decomposing substance moved to the right, causing the sort of curve illustrated. Such a reaction would not be surprising in view of the high acidity of the system used.

The first indication that the form of Curve A might be attributable to the interconversion of several modifications of the same substance was the striking similarity between fractions removed from various tubes of the distribution apparatus.

The concentrations of streptomycin in the run illustrated by Curve A were measured by the maltol test, by bioassay, and by the quantitative application of the Sakaguchi reaction. The modification of the latter suggested by Thomas, Ingalls, and Luck (13) was used as described in the literature, the more concentrated sample being diluted to approximately

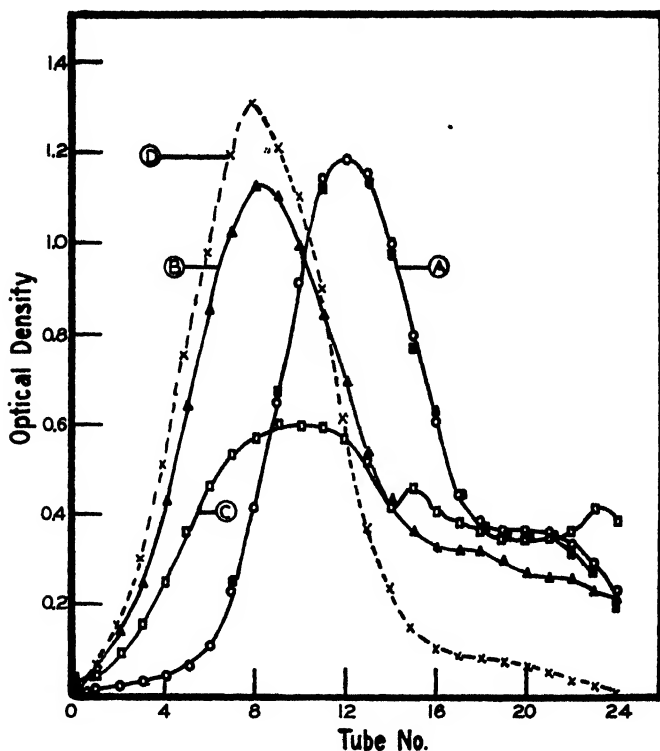


FIG. 1. Distribution curves. Curve A, early chromatographic fraction in butanol and 5 per cent toluenesulfonic acid. ■, the optical density obtained by the Sakaguchi reaction. All other symbols represent the change in optical density at 325  $m\mu$  on heating in alkaline solution. Curve B, distribution of the same material in butanol and 3 per cent toluenesulfonic acid at 22°. Curve C, the same material distributed as in Curve B but at 5°. Curve D, the same material after standing in 0.2 N  $H_2SO_4$ , distributed as in Curve C.

100 units per ml. with the blank lower layer prior to the treatment with concentrated alkali. The correspondence of the maltol and Sakaguchi determinations is illustrated in Curve A in Fig. 1. Bioassay of these fractions against *Klebsiella pneumoniae* (14) revealed that the ratio of biological units per ml. to the optical density at 325  $m\mu$  of the maltol solution produced in the chemical determination was constant, regardless of the fractions



analyzed. The average deviation of  $\pm 5.3$  from the value of 88.2 for the constant was within the limit of error of the microbiological assays, so that no difference in potency, streptidine content, or ability to produce maltol could be demonstrated between the fractions in the higher numbered tubes and those in the central band.

Further evidence was obtained in an attempt to isolate some of the material in the fraction of the higher partition coefficient. A twenty-four plate run with 5 per cent toluenesulfonic acid was made with material with the

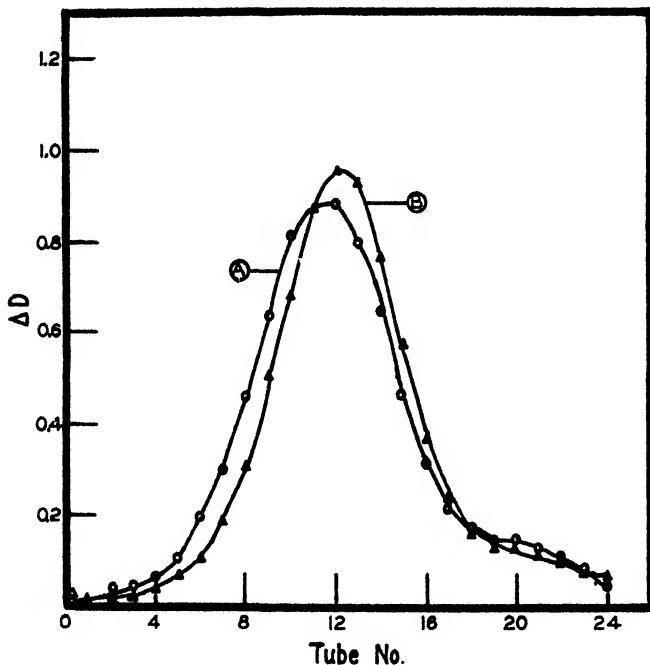


Fig. 2. Distribution curves. Curve A, an early chromatographic fraction; Curve B, redistribution of material obtained from Tube 22 of Curve A.

distribution pattern illustrated in Curve A, Fig. 2. The conditions were identical to those used in obtaining Curve A, but the run was made with 50-fold quantities in 1 liter separatory funnels. The lower layer in the separatory funnel corresponding to Tube 22 of the machine was neutralized with  $\text{Ba}(\text{OH})_2$  and the solution freeze-dried. The solid residue was transferred to a glass-stoppered centrifuge tube and triturated with twice its volume of anhydrous methanol to separate the readily soluble streptomycin trihydrochloride from the relatively insoluble (9.8 mg. per ml.) barium *p*-toluenesulfonate. After centrifugation and removal of the supernatant, the residue was triturated with another portion of methanol and centrifuged.

The combined methanol extracts were evaporated to dryness in the cold and the triturations with methanol were repeated. Approximately 10 per cent of the total streptomycin content was lost at each step. Evaporation of the final methanol solution left a residue of 37 mg. of solid material containing 22 per cent of streptomycin base, calculated from maltol analysis.

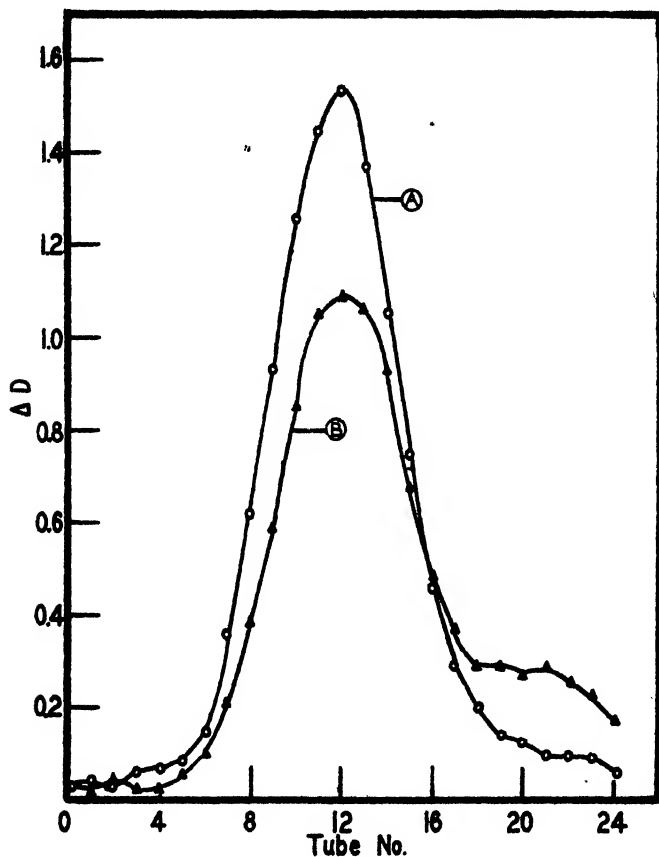


FIG. 3. Curve A, distribution of pure calcium chloride double salt of streptomycin hydrochloride; Curve B, distribution of the same material after standing at pH 7.42.

Redistribution of 20.9 mg. of the material gave Curve B, Fig. 2, practically identical with that of the starting material, a result which could only be explained by transformation of the high  $K$  material into a substance of lower partition coefficient.

In order to determine whether the high  $K$  fraction, which appeared from the above to be unstable in acid, could be reconstituted at higher pH, a

sample of 9.9 mg. of the pure calcium chloride double salt of streptomycin (11) was allowed to stand in solution at pH 7.42 for 30 hours. The solution was freeze-dried and the residue distributed. Fig. 3 illustrates the significant increase in material of high *K* caused by this treatment, Curve A representing the distribution in 5 per cent toluenesulfonic acid of 10 mg. of the original sample and Curve B the same material after standing.

The ready interconvertibility of these fractions strongly suggested that streptomycin can exist in several tautomeric modifications, the forms of high *K* being favored in alkaline and those of low *K* in acid solutions. To determine quantitatively the relative proportions of such substances by distribution in a system in which one form is rapidly being converted to the other is obviously impossible. A qualitative demonstration of their existence is possible only when conversion proceeds slowly enough so that significant quantities of the forms with higher *K* survive to be carried to the higher numbered tubes of the apparatus. The distorted distribution curves caused by this phenomenon likewise make it impossible to state with certainty the number of tautomeric forms.

These difficulties were partially surmounted by the use of less drastic conditions.

It was found possible to slow the rate of conversion of one form to the other by running distributions with 3 rather than 5 per cent toluenesulfonic acid, and to do so even more effectively by using the 3 per cent system at 5°. No other changes were made in the described procedure and the only effect was a lowering of the distribution constant. Distribution in these systems revealed that the early chromatographic fractions contained far higher proportions of the high *K* forms than originally suspected. Curves B and C of Fig. 1 illustrate the patterns obtained when 12 mg. samples of the same material whose distribution is recorded in Curve A were run in the 3 per cent system at 22°, and in the 3 per cent system at 5°.

Use of these systems permitted a clearer demonstration of the interconvertibility of the tautomeric forms. A sample of 12 mg. of the early chromatographic fraction whose distributions are recorded in Fig. 1 was allowed to stand 24 hours in dilute sulfuric acid, neutralized, and freeze-dried. Distribution of the residue in 3 per cent toluenesulfonic acid is recorded as Curve D in Fig. 1, and comparison with Curve B in the same figure makes clear the decrease in the amount of material in Tubes 18 to 24, and the corresponding increase in the centrally located band.

The formation of the high *K* modifications in alkaline solution was strikingly illustrated when distributions were run at 5° with 3 per cent toluenesulfonic acid systems. Curve A in Fig. 4 represents such a distribution of 19.8 mg. of the same sample of calcium chloride double salt which was used for Curve A of Fig. 3. A 24.6 mg. sample of this salt was allowed to stand

in solution at pH 7.52 at room temperature for 24 hours, after which the solution was freeze-dried and the residue distributed as before, with the result shown in Curve B of Fig. 4.

More nearly complete conversion was noted when another sample was dissolved in water, brought to pH 8.77, and allowed to stand for 72 hours, during which time 16 per cent decomposed, as measured by the loss in ability to produce maltol. An aliquot of this material was freeze-dried and the residue distributed at 5° with the 3 per cent system, as shown in Curve B of Fig. 4.

The presence of the aldehyde group in the streptose portion of the mole-

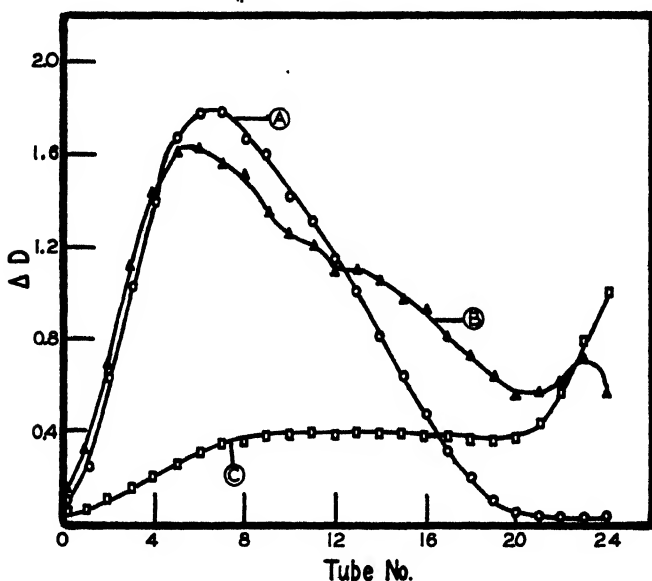


FIG. 4. Distribution in butanol and 3 per cent toluenesulfonic acid at 5°. Curve A, the pure calcium chloride double salt of streptomycin trihydrochloride; Curve B, the same material after standing at pH 7.52; Curve C, the same material after standing at pH 8.77.

cule is apparently essential for these interconversions, since reduction to the dihydro derivative eliminated the tautomerism. The dihydro derivatives of either the early chromatographic fractions or the pure calcium chloride double salt, when distributed in butanol and 5 per cent sulfonic acid, gave identical curves in good agreement with the theoretical distribution of a homogeneous substance. Distributions of 40 mg. samples of these substances are illustrated in Fig. 5.

It seems plausible, then, to suggest that, although the phenomena might

at first glance be taken as evidence for the existence of a third substance related to streptomycin, they are more probably an indication of a tautomeric mixture, possible components of which might be the *aldehydo* structure proposed by Kuehl and coworkers (15), and a ring form obtained by

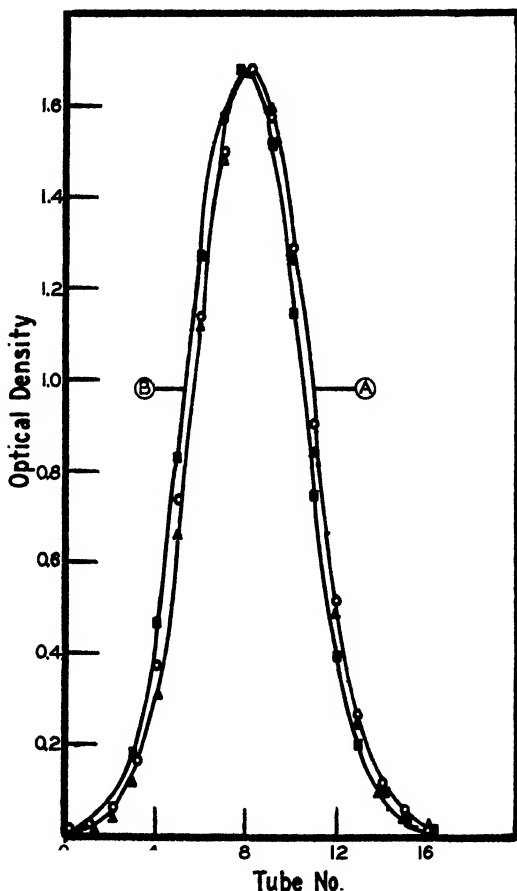


FIG. 5. Distribution curves of dihydrostreptomycin. Curve A, the dihydro derivative from an early chromatographic fraction; Curve B, the dihydro derivative prepared from the pure calcium chloride double salt of streptomycin.  $\Delta$  indicates the theoretical distribution of a homogeneous substance.

the reaction between the aldehyde group and a guanidine group from the streptidine moiety.

There is some evidence for the existence of similar modifications of mannosidostreptomycin since samples of the latter give curves of the same general form as the early chromatographic fractions of streptomycin. Be-

cause of the distribution constant of 0.44 for this substance in the 5 per cent toluenesulfonic acid system, most of the material appears in a band with maximum concentration at Tube 7, while that portion of the curve assumed to represent the form with higher  $K$  occurs in the region of Tube 12. The possibility that this fraction indicates contamination of these samples with streptomycin has been eliminated by distribution in other two-phase systems which differentiate between the streptomycins, but do not distinguish between tautomeric modifications. These systems are described elsewhere by others in these laboratories.<sup>2</sup>

#### EXPERIMENTAL

*Counter-Current Distribution*—The immiscible liquid pairs used for these studies were prepared by mutually saturating *n*-butanol and water. Eastman c.p. *p*-toluenesulfonic acid monohydrate was made up in the water to the desired concentration, usually 3 or 5 gm. per 100 ml., and this solution was then shaken for several minutes with an equal volume of the butanol.

For the preliminary determination of distribution constants in these systems, streptomycin hydrochloride, assaying at 750 units per mg., which had been purified by the chromatographic procedure of Carter *et al.* (10), recrystallization of the helianthate (11), and conversion to the hydrochloride, was made up to 100  $\gamma$  per ml. in the aqueous phase. Equal volumes of this solution and the butanol upper layer were shaken together for 2 minutes and allowed to separate. The streptomycin concentration in the aqueous layer was determined by the maltol reaction before and after shaking and the distribution constant calculated as the difference in the two readings divided by the final concentration.

To determine streptomycin concentrations, 0.2 ml. of 4 *N* NaOH was added to 4 ml. of the aqueous phase in a test-tube. The contents were mixed well, heated in a boiling water bath for exactly 10 minutes, and brought to room temperature by cooling in an ice bath. Loss of volume by evaporation during the heating was prevented by suspending a small funnel in the mouth of each test-tube to provide a surface for condensation and blowing a stream of air against the exposed upper portion of the test-tube. The ultraviolet absorption at 325  $m\mu$  of each solution was measured before and after heating, with 1 cm. cells in the Beckman quartz spectrophotometer, and the difference between the two readings, referred to as  $\Delta D$ , was taken as a measure of the streptomycin concentration. Values of  $\Delta D$  obtained in these toluenesulfonic acid systems are shown in Table I to be proportional to the streptomycin concentration down to levels of approximately 25 units per ml. Below this, the increasing yield of maltol necessitates the use of correction factors to retain the proportionality.

<sup>2</sup> Plaut, G. W., and McCormack, D. R., in press.

The distribution constant of streptomycin varied with the concentration of *p*-toluenesulfonic acid, values of 0.3, 1.0, and 1.8 being obtained with acid made up to 3, 5, and 10 per cent in the aqueous phase.

Partition coefficients in the 3 and 5 per cent systems proved independent of streptomycin concentration within the limits encountered in the distribu-

TABLE I  
*Correction of  $\Delta D$  Values*

Units per ml.	$\Delta D$ per unit per ml. $\times 100$	Correction, per cent of $\Delta D$
100	1.92	
75	2.00	
60	1.95	
50	1.99	
30	2.07	
25	2.02	
15	2.18	-9.0
10	2.25	-11.0
5	2.54	-21.2
2.5	3.04	-34.0
1.25	4.24	-53

TABLE II  
*Distribution Constant of Streptomycin in Butanol-5 Per Cent  
*p*-Toluenesulfonic Acid*

Mg. per ml.	Distribution constant	Per cent recovery by benzene treatment
100	0.124	
50	0.25	
25	0.46	
2.0	1.2	83.9
1.0	1.1	76.4
0.50	1.2	77.5
0.25	1.4	76.5
0.13	1.4	76.5
0.063	1.1	78.8
0.031	1.1	81.5

tions, as illustrated by the series of determinations summarized in Table II.

Distribution curves obtained with 10, 50, and 130 mg. samples of the same material proved superimposable, so that deviations of experimentally obtained curves from the theoretically predicted patterns were not attributable to variations in partition coefficient during the runs.

In spite of the acidity of the systems, samples of streptomycin showed no loss in potency when allowed to stand for 45 hours in these solutions.

All distributions were made in a twenty-four plate Craig counter-current apparatus similar to a previously described model (8), and containing 8 ml. of each phase in each tube. Samples of 8 to 10 mg. were dissolved in 8 ml. of one phase and placed in the 0 tube of the machine. The phases were equilibrated by rocking the apparatus 2 minutes and were allowed to separate for 2 minutes before each movement of the upper half of the machine to a new position.

Although it would have been desirable to displace all of the streptomycin in each tube into the lower layer so that the maltol analysis of the aqueous phase would reveal the total material in each tube, this did not prove practicable. It was possible, however, by the addition to the upper layer of one-half its volume of benzene to displace 26 to 30 per cent of the streptomycin contained in the butanol into the aqueous phase. This was checked experimentally at the time the distribution constants were measured and Table II lists the per cent of streptomycin recovered when a solution in the lower phase was shaken with an equal volume of upper layer and half the volume of benzene.

The variations in  $K$  values listed for the lower streptomycin concentrations in Table II are attributable to the fact that the constant was computed from the formula  $K = (C_B - C_A)/(C_A)$  where  $C_B$  and  $C_A$  represent the concentrations of streptomycin in the lower phase before and after shaking with an equal volume of the upper. Errors in determining concentration thus enter the computation twice and are magnified in the value for  $K$ . The partition coefficient calculated by the method of Williamson and Craig (12) from the distribution curves with the 5 per cent toluenesulfonic acid system averaged 0.97 in runs made over a period of some months, the most extreme variations being from 0.92 to 1.05.

Recoveries of streptomycin from the distribution apparatus were calculated by summation of the  $\Delta D$  values from each tube. These were usually between 75 and 80 per cent in agreement with Table II. In extreme cases in which most of the streptomycin was present as the modification with high partition coefficient (*e.g.* Curve C, Fig. 4) the recoveries were lower, 68 to 70 per cent, as would be expected from the fact that most of the material in the higher numbered tubes is contained in the upper phases, so that the benzene treatment displaces a smaller proportion of the total into the lower layers. The same reasoning accounts for the higher recoveries of 80 to 82 per cent at the other extreme (*e.g.* Curve D, Fig. 1), where most of the streptomycin is concentrated in the fractions of lower  $K$ .

Because of the phenomena herein described the  $K$  values in the 3 per cent system could not be calculated as precisely from the curves, but the



best estimates were from 0.49 to 0.55 at room temperature ( $21 \pm 2^\circ$ ), and from 0.29 to 0.39 at  $5^\circ$ .

**Chromatography of Crude Material**—Through a column 3.2 cm. in diameter containing 460 gm. of alumina was passed a solution of 10 gm. of streptomycin (450 units per mg.) in 100 ml. of methanol. 80 ml. fractions were collected and the solvent removed *in vacuo*. The residues were taken up in 10 ml. of water, filtered, and lyophilized. Table III summarizes a typical experiment. In order to indicate the changing proportions of the forms with low and high partition coefficients, the final column of Table III lists the ratio of material appearing in Tubes 0 to 17 to that found in Tubes 18

TABLE III  
*Chromatography of Crude Streptomycins*

[Fraction	Weight	Biopotency	Ratio of material in Tubes 1-17 Tubes 18-24
	gm.		
A	0.2160	495	2.98
B	0.3186		
C	0.3862	593	
D	0.3559		
E	0.2628	612	4.98
F	0.2758		
G	0.4701	538	
H	0.3616		
I	0.3682	392	10.61
J	0.2917		
K	0.2049	373	
L	0.2219		

to 24 when these chromatographic fractions were distributed in butanol and 5 per cent toluenesulfonic acid.

**Attempted Isolation of Material of High K**—A 40 mg. sample of an early chromatographic fraction was distributed as described in a 5 per cent toluenesulfonic acid system. The lower layers, after the conversion to maltol, were diluted 1:5 with 0.1 N NaOH to obtain the  $\Delta D$  values plotted in Curve A, Fig. 2.

In order to duplicate this run with 40-fold quantities of streptomycin and solvents, the distribution was made in a series of 1 liter separatory funnels, as described elsewhere (9). Since after each equilibration in separatory funnels the lower layer is transferred to the next funnel, those substances of lowest partition coefficient are carried farthest along the line of funnels. This is the reverse of the situation in the Craig apparatus, where the upper layer moves. The two techniques give identical curves if the order of num-

bering the tubes is reversed. For this reason, the funnel to which the sample was added and in which the first equilibration occurred was Tube 24 and succeeding funnels were numbered in descending order. Since only the fractions of the higher distribution constant were desired, it was necessary to set up but three funnels, Tubes 24 to 22. Each contained 320 ml. of the upper layer. The sample was added to Tube 24 and equilibrated, and the lower layer drawn into Tube 23. The fresh lower layer was then added to Tube 24 and both vessels were shaken. The lower phase of Tube 23 was then drawn into Tube 22, that from Tube 24 into Tube 23, etc., the process being repeated until Tubes 24, 23, and 22 had had 24, 23, and 22 equilibrations respectively. The lower phase from Tube 22 was drawn off and discarded at each step. The contents of Tube 22 were shaken with benzene as usual, and the lower layers withdrawn, titrated to pH 6 to 7 with  $\text{Ba}(\text{OH})_2$ , and freeze-dried. The residues were broken up in 15 ml. of dry methanol, shaken vigorously, and centrifuged. The supernatant was removed and the residue again extracted with 10 ml. of methanol.

The combined methanol solutions were evaporated to dryness, and the residue extracted as before with 1.0 and 0.5 ml. portions of methanol. Evaporation of the solvent left a residue weighing 36.6 mg. Over-all recovery of the streptomycin originally in the lower layer was 66 per cent, based on analyses of each fraction by conversion to maltol. 20.9 mg. of the material were distributed with 5 per cent toluenesulfonic acid (Curve B, Fig. 2).

The authors wish to acknowledge the assistance of Mr. Edward Paredes in the chromatographic separations and of Miss Antonine Hoffmann, Mr. Stanley Ulick, and Mr. Robert Matusow in the counter-current distributions.

#### SUMMARY

Evidence obtained by counter-current distribution indicates that streptomycin exists as a mixture of tautomeric modifications, the relative proportions of which depend upon pH. The tautomers are at least partially separable by chromatography.

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## FURTHER STUDIES OF UNIDENTIFIED FACTORS REQUIRED IN THE NUTRITION OF *LACTOBACILLUS CASEI*\*

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Unknown growth factors have been reported for several lactobacilli. Cheldelin and Riggs (1) stated that *Lactobacillus geyonii* 8289 requires a factor in Wilson's liver fraction L for growth on a casein hydrolysate medium containing norit and Lloyd's reagent filtrates of peptone and yeast extract. Metcalf, Hucker, and Carpenter (2) found a growth factor present in several vegetable juices, including carrot, beet, onion, cabbage, and tomato juice. This was a heat-stable factor which caused a stimulation of acid production by certain lactobacilli. Kuiken, Norman, Lyman, Hale, and Blotter (3) reported an unknown factor in tomato juice necessary for the growth of *Lactobacillus arabinosus*. This substance was adsorbed by norit. Shorb (4, 5) reported an unidentified growth factor for *Lactobacillus lactis* Dorner in refined liver extracts used in treating pernicious anemia, and one in clarified tomato juice. Both of these factors were required for optimum growth of this organism. The liver factor was found to be concentrated in the refined extracts in practically a linear relationship to the potency of their antianemic activity.

Tomato juice has been known to contain a factor or factors needed for the optimum growth of many microorganisms. Grossowicz (6) in 1942 reported that tomato juice contained a new factor, adsorbable on norit, which produced good growth of *Streptococcus hemolyticus*, type A.

Woolley (7) reported the existence of an unknown growth factor in liver for hemolytic streptococci, and later Sprince and Woolley (8) found it to be necessary for the growth of *Lactobacillus casei*. The active material, which they named strepogenin, was found in largest amounts in crystalline insulin, trypsinogen, trypsin, and casein (9). On a medium containing casein hydrolysate that had been hydrolyzed with  $H_2SO_4$  to destroy the strep-

\* This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D. C., and was aided by grants to Cornell University by the Cerophyl Laboratories, Inc., Kansas City, Missouri, the Nutrition Foundation, Inc., New York, and the Western Condensing Company, San Francisco, California. This work was conducted in the Nutrition Laboratories of the Department of Poultry Husbandry.

ogenin activity, *Lactobacillus casei* would grow when strepogenin concentrates were added as the only supplement.

Scott, Norris, and Heuser (10) found that two unknown factors were required for the growth of *Lactobacillus casei*. One of these factors was usually associated with products of animal origin, and the other was present in the Darco filtrate of tryptone. The unadsorbable factor was referred to as strepogenin, although, contrary to the work of Woolley, this substance, when added to a casein hydrolysate basal medium, did not promote growth. Only in the presence of the so called "animal protein factor" did maximum growth occur. Scott, Norris, and Heuser (11) have correlated factor S activity of several feedstuffs for the chick with their strepogenin activity for *Lactobacillus casei*. Their results demonstrate that the same activity is being measured by the microorganism and the chick.

In view of these findings and recent work by Nichol, Robblee, Cravens, and Elvehjem (12) on the effect of liver extract on the growth of chicks, a study was made to determine the response of *Lactobacillus casei* to liver extract and tomato juice.

#### EXPERIMENTAL

*Culture*—The culture of *Lactobacillus casei* was obtained from the American Type Culture Collection. It was maintained in stab culture in a medium containing liver, glucose, yeast, tryptone, and agar, the composition of which is described by Nymon, Gunsalus, and Gortner (13). Weekly transfers were made through broth cultures of the same composition, except for the omission of agar.

The inoculum tubes were prepared by adding tryptone (adsorbed  $\frac{1}{2}$  hour with an equal weight of Darco G-60) at a level of 25 mg. per tube to the basal medium. The organism was grown for 18 to 24 hours in this inoculum tube at 37°. It was then transferred to a sterile centrifuge tube and centrifuged for 15 minutes at approximately 2500 R.P.M. The medium was discarded and the organisms were resuspended in sterile saline, and diluted until a reading of between 60 and 70 was obtained on the galvanometer of a Coleman spectrophotometer set at a wave-length of 650 m $\mu$ . One loopful of this suspension was used to inoculate each assay tube.

*Medium Used*—An amino acid mixture was used as the nitrogen source in the medium instead of casein hydrolysate. The amino acids used (and the amounts of each) were the same as those given in the report by Stokes, Gunness, Dwyer, and Caswell (14) for *Streptococcus faecalis*. This mixture contained all sixteen amino acids shown by Hutchings and Peterson (15) to be needed by *Lactobacillus casei*, and in addition glycine, proline, hydroxyproline, and norleucine. The other constituents of the medium were the same as those reported by Scott, Norris, and Heuser (10) with one ex-

ception. The pyridoxal was added to the medium before sterilization at a level of 10  $\gamma$  per tube. The assay tubes were steamed for 15 minutes, cooled to 35–40°, and inoculated. They were incubated at 37° for 16 to 18 hours. At the end of this time the turbidity of the suspensions was read in a Coleman spectrophotometer at a wave-length of 650  $m\mu$ .

*Strepogenin Concentrate from Casein*—The source of strepogenin used in this study was a concentrate made from Labco or Difco vitamin-free casein by the method of Sprince and Woolley (9) as modified by Scott.<sup>1</sup> It was prepared as follows: 1 liter of distilled water was added to 100 gm. of casein. (In the case of Labco casein the suspension was steamed 3 minutes and cooled.) The pH was adjusted to 8.0 with NaOH. 2 gm. of trypsin (Pfanstiehl) were added to the mixture, which was covered well with toluene, and allowed to digest at 37° for 20 hours. It was necessary to adjust the pH several times in order to keep it at 8. After 20 hours the pH was changed to 7.0 and the insoluble material was filtered off. 50 gm. of lead acetate were added and the resulting precipitate was removed by filtration. The pH was then adjusted to 9.5 and another precipitate filtered off. The filtrate was saturated with H<sub>2</sub>S and the PbS was removed by filtration. The filtrate was concentrated under a vacuum until all of the H<sub>2</sub>S was removed. At this point the activity of the strepogenin concentrate was tested with *Lactobacillus casei*. If growth occurred when the strepogenin concentrate was added to the basal medium, the concentrate was treated with an equal weight of norit for  $\frac{1}{2}$  hour, or until no growth occurred when it was the only supplement to the medium.

*Liver Extracts*—The liver extracts tested were crude and refined preparations used in the treatment of pernicious anemia. Six different extracts containing 15 U. S. P. units per ml. were studied, along with one each of 2, 5, and 10 U. S. P. units per ml. All of the original preparations were secured from a local pharmacy. When a larger amount of one extract was needed, it was supplied by the manufacturer directly.

*Tomato Juice*—Five samples of canned tomato juice were purchased on the open market early in October. Each was filtered through No. 44 Whatman filter paper on a Büchner funnel with the aid of Celite filter aid. The resulting solutions were yellow in color. Part of each solution was adsorbed with norit at the natural pH of the serum (4.0 to 4.2) for  $\frac{1}{2}$  hour (5 gm. of norit per 200 ml. of serum) and filtered to remove the charcoal. The resulting solution was colorless.

#### RESULTS AND DISCUSSION

In the first study, varying levels of strepogenin were added to increasing levels of liver extract. The results of this study are presented in Table I.

<sup>1</sup> Scott, M. L., unpublished data, Cornell University, Ithaca, New York.

When streptogenin or liver extract was added alone to the basal medium, no growth occurred. However, the addition of both together resulted in maximum growth. It is evident that streptogenin equivalent to 25 mg. of original casein per tube was enough to supply the streptogenin requirement of *Lactobacillus casei* in this case. It was necessary to establish this level for each new streptogenin preparation. Different batches of the liver extract gave relatively uniform responses.

It is evident that a clear cut differentiation in factors has been obtained. The unadsorbed activity in trypsin-digested casein, which is not precipi-

TABLE I

*Effect of Streptogenin and Liver Extract on Growth of Lactobacillus casei*  
17 hour incubation.

Streptogenin*	APF†	Galvanometer reading‡
mg. per tube	unit per tube	
0	0	100
0	0.4	90
50	0	100
10	0.1	75
10	0.2	40
10	0.3	26
10	0.4	23
25	0.1	40
25	0.2	20
25	0.3	17
25	0.4	16
50	0.1	41
50	0.2	24
50	0.3	19
50	0.4	15

\* Streptogenin concentrate from Labco vitamin-free casein.

† Lilly's antipernicious anemia extract, 15 units per ml.

‡ A galvanometer reading of 100 represents no growth.

tated by lead, is identified as streptogenin. The activity present in the liver extract shall henceforth be called "animal protein factor" (APF) since unpublished data<sup>2</sup> indicate that this activity is correlated with the response of the chicks fed an all vegetable diet to preparations of the animal protein factor.

Having established the fact that liver extracts contain a growth factor for *Lactobacillus casei*, it was considered advisable to test the activity of different liver preparations. Eight more extracts were assayed for APF activity with streptogenin in the medium at a level equivalent to 25 mg. of original casein per tube. The results of this study are presented in Table II.

<sup>2</sup> Combs, G. F., unpublished data, Cornell University.

TABLE II

*Response of Lactobacillus casei to Various Liver Extracts in Presence of Strepogenin\**  
18 hour incubation.

APF	Level, units per tube	Galvanometer reading†	Activity index‡
None		100	
Lilly, 15 units per ml.§	0.05	57	100
	0.10	27	
	0.20	12	
	0.30	10	
Sample A,   5 units per ml.	0.10	48	56
	0.20	27	
	0.30	16	
	0.40	11	
" B, 15 " " "	0.10	65	39
	0.20	41	
	0.30	25	
	0.40	13	
" C, 15 " " "	0.25	39	25
	0.50	21	
	0.75	16	
	1.00	11	
" D, 2 " " "	0.25	68	14
	0.50	47	
	0.75	26	
	1.00	15	
" E, 15 " " "	0.25	85	10
	0.50	58	
	0.75	33	
	1.00	21	
" F, 15 " " "	0.50	66	7
	1.00	54	
	1.50	23	
	2.00	14	
" G, 15 " " "	0.50	78	6
	1.00	59	
	1.50	29	
	2.00	19	
" H, 10 " " "	1.00	65	4
	2.00	47	
	3.00	17	
	4.00	9	

\* Strepogenin concentrate from Labco vitamin-free casein at the level of 50 mg. per tube.

† A galvanometer reading of 100 represents no growth.

‡ The activity index has been obtained by comparing the activity of APF with the activity of Lilly's extract, which has been assigned a value of 100.

§ Potency of extracts as purchased.

|| Extracts from different pharmaceutical companies.



It is evident from the data that there are great differences in the response of *Lactobacillus casei* to these nine liver extracts. The variations obtained do not seem to be related to the degree of refinement of the preparation, since the most active preparation had a concentration of 15 units of antipernicious anemia factor per ml., and three of the least active extracts had the same concentration. A crude sample (D) had an intermediate activity. According to the United States Pharmacopeia XIII (16) the U. S. P. unit of antipernicious anemia activity is an amount of product which, when administered daily, produces clinical and hematological responses in Addisonian pernicious anemia as judged by the Advisory Board to be satisfactory. Any standardization made with human patients is subject to some variation, but it is hardly likely that such wide differences in liver extracts would be caused by variations in standardization. Therefore, it would seem impossible that this APF activity for *Lactobacillus casei* is identical with the factor required in the treatment of pernicious anemia. However, there is a strong possibility that *Lactobacillus casei* requires two factors. One of these may be identical with the antipernicious anemia factor and the other present in varying amounts in the different liver extracts.

In the study with tomato juice the untreated serum was added to the basal medium, and the adsorbed serum was added to the medium alone and in combination with liver extract. The results of this study are presented in Table III.

It is evident from the data that there are two factors present in filtered tomato juice. One of these is rather easily adsorbed by norit and is probably the same as the growth-promoting factor (APF) present in liver extracts. The factor that is not readily adsorbed by charcoal when added with liver extract produces a response in *Lactobacillus casei* that is similar to the effect of strepogenin.

In order to investigate the relationship between the strepogenin concentrate from casein and adsorbed tomato juice, a study was made with these two preparations with graded levels of liver extracts. The results of this study are presented in Table IV.

The factor that is not adsorbed from tomato juice by norit under these conditions has the same growth-promoting activity as the strepogenin concentrate made from casein. This would indicate that the substance in tomato juice is either identical to strepogenin from casein or very similar to it.

Since these studies have been performed, several samples of tomato juice have been found to contain considerably less of the "animal protein factor." These cans have been standing on the shelves for several months, and it is possible that loss of this activity has occurred, due to instability. There

was no difference in this respect between tomato juice canned in metal and that canned in glass.

The results of these studies demonstrate that there are two or possibly

TABLE III

*Growth Response of Lactobacillus casei to Tomato Juice and Liver Extract*  
18 hour incubation.

Supplement	Level	Galvanometer reading*	Supplement	Level	Galvanometer reading*
	ml.			ml.	
None		100	Tomato juice, Sample B (adsorbed)	4	90
Tomato juice, Sample A†	1	25	APF (0.5 unit) + tomato juice, Sample B (adsorbed)	1	17
	2	9		2	10
	3	9		3	8
" " " B	1	22	Tomato juice, Sample C (adsorbed)	4	83
	2	13			
	3	9	APF (0.5 unit) + tomato juice, Sample C (adsorbed)	1	18
" " " C	1	20		2	10
	2	9		3	8
	3	8			
" " " D	1	37	Tomato juice, Sample D (adsorbed)	4	91
	2	23			
	3	17	APF (0.5 unit) + tomato juice, Sample D (adsorbed)	1	17
	4	7		2	11
				3	9
" " " E	1	23	Tomato juice, Sample E (adsorbed)	4	94
	2	10			
	3	8	APF (0.5 unit) + tomato juice, Sample E (adsorbed)	1	31
	unit			2	18
APF‡	0.5	90		3	9
	ml.				
Tomato juice, Sample A (adsorbed)§	4	97			
APF (0.5 unit) + tomato juice, Sample A (adsorbed)	1	29			
	2	9			
	3	8			

\* A galvanometer reading of 100 represents no growth.

† Filtered tomato juice of five different brands.

‡ 15 units per ml. of Lilly's liver extract.

§ Serum adsorbed  $\frac{1}{2}$  hour with norit (5 gm. per 200 ml.).

three unidentified factors necessary for the growth of *Lactobacillus casei*. One of these is streptogenin and the other is a factor or factors associated with animal proteins and found in high concentration in certain liver ex-

tracts used for the treatment of pernicious anemia. In the work of Sprince and Woolley (8) with *Lactobacillus casei* the casein hydrolysate used in the basal medium may have contained sufficient APF activity for the organism. On the other hand, it is possible that their trypsin-digested strepogenin concentrates supplied this activity, since we have found trypsin to be an excellent source of APF. It is possible that the factors of Cheldelin and Riggs (1), Grossowicz (6), and Kuiken *et al.* (3) are the same as the liver factors reported here. There is little doubt that the factors required by *Lactobacillus lactis* Dorner, as described by Shorb (4, 5), are identical with strepogenin and APF. The question of whether each of these activities is

TABLE IV

*Comparison of Strepogenin Concentrate from Casein and Adsorbed Tomato Juice As Growth Factor for Lactobacillus casei*

Supplement	Level	Galvanometer reading*
None		100
APF†	0.4 unit	90
Tomato juice (adsorbed)‡	4 ml.	96
Strepogenin concentrate§	50 mg.	96
“ “ (50 mg.) + APF	0.1 unit	41
	0.2 “	24
	0.3 “	19
	0.4 “	15
Tomato juice (adsorbed) (4 ml.) + APF	0.1 “	49
	0.2 “	26
	0.3 “	18
	0.4 “	12

\* A galvanometer reading of 100 represents no growth.

† 15 units per ml. of Lilly's liver extract.

‡ Tomato juice serum adsorbed  $\frac{1}{2}$  hour with norit (5 gm. per 200 ml.).

§ Strepogenin concentrate from Labco vitamin-free casein.

represented by a single factor is not known. This problem is now under investigation in this laboratory.

#### SUMMARY

Studies have been conducted showing that *Lactobacillus casei* requires a factor or factors (APF) present in antipernicious anemia liver extracts, in addition to strepogenin, for maximum growth. Wide variations were found in the amount of factor (or factors) present in liver extracts, which is not attributable to differences in their antipernicious anemia activity. These differences may result from the presence of the growth factor as a contam-

inant of the antipernicious anemia preparations, or from the existence of two factors in varying amounts in the several liver extracts.

Tomato juice, shortly after the canning season, contained all of the unidentified growth factors for *Lactobacillus casei* in rather large amounts. Adsorption of tomato juice by norit removed the APF activity, but did not alter the streptogenin activity which was recovered in the filtrate. The APF activity in tomato juice decreased as the age of the canned product increased, whereas the streptogenin activity of the tomato juice was unaltered.

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# THE SYNTHESIS OF PROTEIN BY NON-PROLIFERATING ESCHERICHIA COLI

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Within the last year several investigations on the uptake of labeled amino acids by the proteins of tissue slices have been described in the literature. This incorporation has been used to indicate the synthesis of new protein, and the technique developed has made it possible to study the effects of various conditions and substances on the synthesis of peptide bonds. Thus Melchior and Tarver (1) have followed the uptake of radio-sulfur, introduced in the form of methionine labeled with  $S^{35}$ , by liver slices, and have found that the incorporation into protein is abolished by homogenizing the tissue. This has been verified in intestinal sections by Winnick, Greenberg, and Friedberg (2), who have shown further that azide ion is inhibitory. Frantz, Loftfield, and Miller (3), in turn, have demonstrated that protein synthesis in liver slices requires the presence of oxygen.

One disadvantage to the use of tissue slices is the considerable spread of results usually observed, due to differences between individual slices. Furthermore, in the use of methionine to measure protein synthesis in liver, the procedure is complicated by the rapid production of labeled cystine or cysteine by the tissue (1). The use of bacteria has suggested itself as a means of overcoming the first difficulty, since the presence of several billions of organisms would tend to minimize individual variations. Since it has been found, in addition, that non-proliferating *Escherichia coli* converts less than 3 per cent of the labeled methionine to cystine or cysteine, and none, within experimental error, to sulfate, the use of microorganisms has seemed especially promising for studying the synthesis of peptide bonds as isolated from the more complex process of growth.

## EXPERIMENTAL

The *Escherichia coli* cells used in these experiments were grown for 18 hours on Bacto-nutrient agar. They were washed off the agar slants with normal saline, washed three times with saline at the centrifuge, then resuspended in saline, precautions to maintain sterile conditions being observed throughout. The suspensions were pipetted into centrifuge tubes containing the labeled methionine and the other substances being investigated, and the tubes were incubated for 6 hours at 37°. Turbidity was measured at the beginning and end of each experiment by a Hellige-Diller photo-

electric colorimeter. The original transmission of light was usually between 60 and 70 per cent and there was generally an increase of 2 to 3 per cent during the incubation; *i.e.*, the number of cells present always decreased during these experiments. No attempt was made to keep the original turbidity constant in different experiments, since controls were run with each experiment. The suspensions contained about 15 mg. of dry weight of bacteria.

At the end of the experiment the tubes were heated to 100° for 20 minutes. The coagulated cells were centrifuged and washed four times with an acetate buffer of pH 5.4. The first wash was permitted to stand overnight to insure removal of any adsorbed methionine. The preparation of samples for analysis has been described by Melchior and Tarver (1). Carrier sulfate was added to make a total precipitate of 0.026 mm. The radioactivity was determined with a Lauritsen electroscope modified in the manner described by Henriques, Kistiakowsky, Margnetti, and Schneider (4).

The penicillin G was a purified sample with a potency of 1630 units per mg.

#### RESULTS AND DISCUSSION

It has been found that a suspension of *Escherichia coli* incubated with labeled methionine incorporates a small but significant amount of the labeled sulfur into the bacterial proteins. A typical experiment is shown in Table I. The number of bacteria present is expressed as 100 minus per cent transmission of light, which is directly proportional to the turbidity. About 0.7 per cent of the original activity is found present in the proteins, although there is a net decrease in turbidity and hence in the number of microorganisms.

It was necessary at the outset to make certain that the uptake of labeled sulfur represents a true synthesis of protein rather than an adsorption phenomenon. Hence one of the bacterial suspensions was transferred to a dialysis bag at the end of the incubation and dialyzed for 42 hours against running tap water. A control sample was handled as described above. The results of this experiment are recorded in Table II. Though there was a decrease in the labeling of the bacteria upon dialysis, the major portion of the activity was not dialyzable. Furthermore, heating the bacteria to 100° completely stops the uptake of radioactivity by the protein. The reaction is also stopped by small amounts of certain inhibitors, as will be described later. Hence the uptake of activity by the bacteria is an enzymatic process whereby the amino acid methionine is firmly attached to large molecules, and this technique can be used as a measure of the amount of protein synthesis occurring during the experiment.

*Effect of Asparagine, Glucose, and Glycine*—The control samples in Table III show that less than 1 per cent of the methionine added as a substrate was incorporated into protein by the resting bacteria. Various substances, including nitrogen, phosphorus, and carbon sources, were added, therefore, in an attempt to increase the synthesis. Asparagine, which is a growth factor of *Escherichia coli*, was found to cause a definite increase in the incorporation of methionine. That this effect is not simply due to the addition of a carbon or nitrogen source is shown by the observation that glycine

TABLE I

*Uptake of Methionine by Non-Proliferating Bacteria*

Washed *Escherichia coli* suspended in 50 ml. of saline. Incubated 6 hours at 37°. 4.0 micromoles of labeled methionine added.

Original turbidity*	Final turbidity*	Labeled S found in proteins
		<i>micromoles</i> $\times 10^3$
44	37	2.9
44.5	37.5	2.9

\* 100 minus per cent transmission of light.

TABLE II

*Test of Enzymatic Nature of Uptake*

Washed *Escherichia coli* suspended in 50 ml. of saline containing 200 mg. per cent of asparagine. Incubated 6 hours at 37°.

Conditions	Labeled methionine added	Labeled S found in proteins
	<i>micromoles</i>	<i>micromoles</i> $\times 10^3$
Control.....	9.4	5.8
Dialyzed 42 hrs.....	9.4	4.4
Control.....	3.0	4.1
Boiled before incubation.....	3.0	0.0

causes a much smaller increase. Glucose not only failed to increase protein synthesis, but caused a definite decrease of about 40 per cent. Thus glucose alone does not support protein synthesis; in fact, when the cells are metabolizing glucose there is a decrease in the production of protein. These data are not sufficiently complete to attribute the stimulation exhibited by asparagine to a specific effect of the growth factor, but it is apparently not due simply to its carbon or nitrogen content.

*Effect of Azide, Cyanide, and Fluoride*—As is recorded in Table IV, azide, cyanide, and fluoride ions markedly inhibit protein synthesis in our system.



Azide and cyanide are known to inhibit enzymes containing iron porphyrin groups such as cytochrome oxidase and catalase. It is pertinent to point out that Frantz, Loftfield, and Miller (3) have found that protein synthesis

TABLE III

*Effect of Asparagine, Glycine, and Glucose*

Washed *Escherichia coli* suspended in 50 ml. of saline. Incubated 6 hours at 37°.

Additions	Labeled methionine added	Labeled S found in proteins
	<i>micromoles</i>	<i>micromoles × 10<sup>2</sup></i>
None.....	4.0	2.9
" .....	4.0	2.9
100 mg. asparagine.....	4.0	4.1
100 " " .....	4.0	4.3
None.....	4.0	2.2
100 mg. asparagine.....	4.0	2.9
100 " " .....	4.0	3.2
100 " glucose.....	4.0	1.4
100 " " .....	4.0	1.3
100 " glycine.....	4.0	2.5
100 " " .....	4.0	2.4
None.....	5.4	1.5
100 mg. glucose.....	5.4	0.9
100 " asparagine.....	5.4	2.8

TABLE IV

*Effect of Inhibitors*

Washed *Escherichia coli* suspended in 50 ml. of saline containing 200 mg. per cent of asparagine. Incubated 6 hours at 37°. 2.2 micromoles of labeled methionine added.

Inhibitor	Labeled S found in proteins	Inhibitor	Labeled S found in proteins
	<i>micromoles × 10<sup>2</sup></i>		<i>micromoles × 10<sup>2</sup></i>
None	1.3	$6.7 \times 10^{-3}$ M NaCN	0.0
"	1.3	$6.7 \times 10^{-3}$ " "	0.1
$5 \times 10^{-3}$ M NaN <sub>3</sub>	0.0	0.1 M M NaF	0.0
$5 \times 10^{-3}$ " "	0.1	0.1 M " "	0.1

in liver is dependent upon a supply of oxygen, and thus it is possible that the synthesis of peptide bonds is directly coupled with an energy-yielding oxidation. Lipmann (5) has suggested that the energy for protein synthe-

sis may be supplied by adenosine triphosphate (ATP), as has proved to be the case in many other endergonic biosyntheses (5-7). Thus ATP may react to form an amino acid phosphate and adenosine diphosphate (ADP), and the amino acid phosphate may then couple with another amino acid to form a peptide and  $H_2PO_4$ , the net reaction being



Since the hydrolysis of ATP is accompanied by a free energy change of about -12,000 calories per mole and the energy required for the synthesis of a peptide bond is probably around 3000 calories per mole (8), such a mechanism would provide the necessary drive for the synthesis of protein. In fact, Borsook and Dubnoff (9) have presented evidence that such a mechanism is responsible for the biosynthesis of the peptide hippuric acid by liver homogenates.

We have observed that  $10^{-3}$  M ATP causes a small increase in protein synthesis in our system, but the results were not outside of experimental error. However, the inhibition by azide and cyanide is support for this mechanism. In line with this point of view one may attribute the inhibition exhibited by glucose to the large excess of glucose acting as a phosphate acceptor and thereby draining the supply of high energy phosphates. Such would be the case if glucose were being converted to glycogen rather than undergoing oxidation to pyruvate. Certainly this aspect of protein synthesis is worthy of further investigation.

Fluoride usually inhibits enzymes which are activated by calcium or magnesium. The inhibition exhibited by this substance calls attention to the fact that when the *Escherichia coli* cells are washed and resuspended in saline the salt content is probably decreased below normal by diffusion into the medium. It would be of interest to examine the effects of various important salts on the synthesis of protein.

*Effect of Penicillin G*—Penicillin G was found to have a marked effect on the growth of heavy suspensions of *Escherichia coli* in a complete synthetic medium (10). Typical growth curves are shown in Fig. 1. In the presence of penicillin, after a slight initial growth there is a marked decrease in the number of cells present. The dotted line shows the "growth" curve for a saline suspension with or without penicillin. The striking effect of penicillin on the number of bacteria was not detected in the non-growing system.

Table V shows the effect of penicillin on protein synthesis. A small and barely significant decrease, of the order of 12 per cent, was observed in the uptake of methionine. These results indicate that penicillin must exert its effect on some aspect of growth other than protein synthesis, at least as indicated by the incorporation of methionine.

**Effect of Sulfanilamide**—In a heavy suspension such as was used in this work, it was difficult to demonstrate any effect of sulfanilamide on growth. Furthermore it must be considered that methionine is one of the substances which antagonizes sulfanilamide. Fig. 2 shows that 300 mg. per cent of sulfanilamide will inhibit the growth of such a suspension, although not completely, and that methionine in the concentration used partially reverses this effect. As is illustrated by Table VI, 300 mg. per cent of sulfanilamide cause a roughly equivalent inhibition of protein synthesis in the non-growing bacteria of between 20 and 40 per cent, an amount sufficient to account for its effectiveness as a bacteriostatic agent under these conditions.

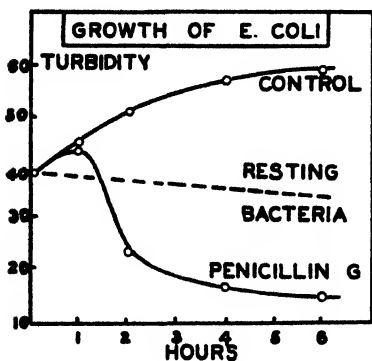


FIG. 1

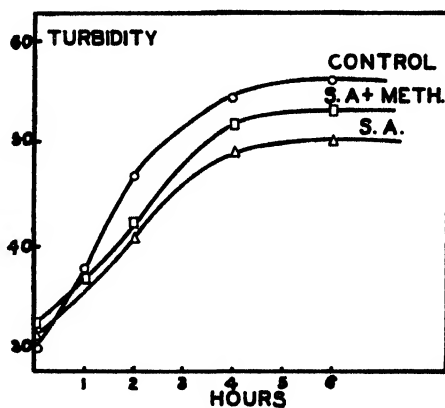


FIG. 2

FIG. 1. Effect of crystalline penicillin G on the growth of *Escherichia coli* in a nutrient medium. The dotted line shows "growth" in saline suspension, with or without penicillin.

FIG. 2. Effect of sulfanilamide and of methionine + sulfanilamide on the growth of *Escherichia coli* in a nutrient medium.

If this is the mechanism of action of sulfanilamide, however, *p*-amino-benzoic acid (PAB) should reverse the inhibition of protein synthesis, since it causes a complete reversal of the effect of sulfanilamide on growth in the complete nutrient medium. For example, 6 mg. per cent of PAB added to the sulfanilamide-inhibited culture in Fig. 2 give a curve closely following the normal control. However, PAB in these concentrations in the saline suspensions proved to exert a definite inhibition on the synthesis of protein, and when added simultaneously with sulfanilamide, the inhibition of protein synthesis was much greater than with sulfanilamide alone.

A similar result has been reported by Clifton and Loewinger (11), who have found that the oxygen uptake of washed *Escherichia coli* in the pres-

ence of glucose or amino acids is inhibited by sulfanilamide. They were unable to reverse this effect with PAB, and report in fact that PAB was inhibitory itself.

Thus the inability of PAB to reverse the effect of sulfanilamide on protein synthesis would indicate, at first glance, that this step is not the one which

TABLE V

*Effect of Penicillin on Protein Synthesis*

Preparation as in Table IV. 4.0 micromoles of labeled methionine added.

Inhibitor	Labeled S found in proteins
	micromoles $\times 10^2$
None	2.9
"	3.2
10 mg. penicillin	2.3
10 " "	2.6

TABLE VI

*Effect of Sulfanilamide and p-Aminobenzoic Acid*

Additions	Labeled methionine added	Labeled S found in proteins
	micromoles	micromoles $\times 10^2$
None	8.6	3.6
"	8.6	3.9
300 mg. % sulfanilamide	8.6	2.2
300 " % "	8.6	2.0
None	5.4	2.4
"	5.4	2.2
300 mg. % sulfanilamide	5.4	2.0
300 " % "	5.4	1.8
300 " % " + 6 mg. % PAB	5.4	1.0
300 " % " + 6 " % "	5.4	0.8
None	5.7	4.0
"	5.7	3.3
6 mg. % PAB	5.7	1.7
6 " % "	5.7	2.2

the sulfonamides block in the growth process. However, it is necessary to recognize the difference between non-proliferating washed cells in a saline suspension and the growing microorganisms in a nutrient medium. In the latter situation, PAB is probably converted to another metabolite, such as a pterioic acid derivative, in which form it counteracts the effect of the sul-

fonamide. The saline suspensions may be unable to convert it to the required metabolite, and it is possible that PAB is itself inhibitory. Until the rôle of PAB is clarified, it is not possible to say that the inhibition of protein synthesis exhibited by sulfanilamide is also the essential step in its bacteriostatic action.

#### SUMMARY

It has been demonstrated that washed cells of *Escherichia coli* incorporate methionine labeled with  $S^{35}$  into non-dialyzable molecules by an enzymatic reaction. This synthesis of protein occurs despite a net decrease in the number of cells present.

The growth factor asparagine has been shown to increase the synthesis. Glycine exhibits a much smaller increase, and glucose causes a definite inhibition.

Azide, cyanide, and fluoride inhibit protein synthesis markedly.

Penicillin G shows a very small inhibition, insufficient to explain its striking effect on the growth of *Escherichia coli* at corresponding concentrations.

Sulfanilamide causes an inhibition of protein synthesis in this system to a degree approximately equal to its effect on growth. However, the addition of *p*-aminobenzoic acid does not reverse the inhibition of protein synthesis in saline suspensions contrary to its effect on growth in nutrient media. In fact, *p*-aminobenzoic acid is itself inhibitory to protein synthesis in the non-growing system.

We are indebted to Dr. Harold Tarver for the labeled methionine used in this investigation, and to Dr. D. W. MacCorquodale of the Abbott Laboratories for a gift of the penicillin G.

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# THE PANTOTHENIC ACID CONTENT OF COENZYME A BY CHICK ASSAY\*

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Lipmann, Kaplan, Novelli, Tuttle, and Guirard (1) reported that, whereas concentrates of the coenzyme required for acetylation (coenzyme A) showed no pantothenic acid activity by the ordinary microbiological assay, considerable amounts of  $\beta$ -alanine were found after acid hydrolysis. From this early observation, it was suspected that the coenzyme might contain combined pantothenic acid which was unavailable to *Lactobacillus cqsai*. If such were the case, it seemed likely that chicks or other animals would be able to utilize this pantothenic acid; the animal would presumably have the mechanism for both the decomposition to, and the synthesis from, the constituent parts. An early trial yielded promising results, but, before sufficient material was available for adequate study, the above authors clearly demonstrated by other means that pantothenic acid was a constituent of the coenzyme (1).

Lipmann, Kaplan, and Novelli (2) have shown that the pantothenic acid appears to be bound by two linkages, one of which is to phosphate, as is indicated by the activity of phosphodiesterase in liberating phosphate with simultaneous loss of coenzyme activity. Both linkages are essential for activity and both must be split before the pantothenic acid becomes "free" when tested by microbiological assay. This is accomplished by intestinal phosphatase and a pigeon liver enzyme together.

It was of considerable interest therefore to determine the availability of the pantothenic acid in coenzyme preparations for the chick, since it now appears that much of the pantothenic acid in certain foods is present as the coenzyme. We have compared the potency of two preparations when administered both orally and intraperitoneally. For comparative purpose the activity of calcium pantothenate given by these two routes was determined.

## EXPERIMENTAL

Day-old white Leghorn cockerels were used in all of these studies. They were fed a commercial chick mash for 4 days and then given a purified diet low in pantothenic acid. This diet was the same as that used by Hegsted

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and Perry (3) with the single exception that only 5 per cent of corn oil was added rather than 10 per cent. After 4 or 5 days, when growth had ceased in most of the chicks, they were divided into comparable groups containing five chicks each and given daily supplements as indicated below. Oral doses were introduced directly into the crop with a graduated pipette.

Calcium pantothenate solutions were made freshly every 4 or 5 days in water to contain 100  $\gamma$  of calcium pantothenate per ml. Coenzyme solutions were made every 2nd day to equal this pantothenic acid concentration according to the results of microbiological assay, sufficient  $\text{NaHCO}_3$  being added to cause complete solution. All solutions were kept in the refrigerator.

Of the three assays reported, Assay 1 was completed in 10 days and Assay 2 and Assay 3 in 8 days. The gain in weight for each chick during this period was calculated by averaging the weights of each chick for the last 3 days of the study and subtracting the original weight. This gives somewhat more consistent results than simply using the weight found on the last day, since daily gains are usually erratic.

The chicks which received *oral* doses of calcium pantothenate were considered to be the standard to which results with other supplements were compared. A smooth curve was drawn through the mean weight of these groups and the relative pantothenic acid activity of the other supplements read from this curve.

Two coenzyme preparations were tested. Preparation 1 contained 23  $\gamma$  of pantothenic acid by microbiological assay and 35 units of coenzyme activity per mg. (4). This was approximately 3 times as potent as Preparation 2 which was found to contain 8.2  $\gamma$  of pantothenic acid and 14 units of coenzyme activity per mg. Neither preparation contained free pantothenic acid for *Lactobacillus casei*.

### Results

The data from three assays in which the coenzyme preparations and calcium pantothenate were compared by two methods of administration are shown in Table I. The results may be briefly summarized. The administration of coenzyme intraperitoneally resulted in the gains expected from the pantothenic acid content determined by microbiological assay; that is, the chick assay agreed with the microbiological assay and apparently all of the pantothenic acid was available to the chick.

In parallel experiments the oral doses yielded considerably smaller gains than were expected, indicating that the chicks were able to utilize only from 43 to 85 per cent of the pantothenic acid in the coenzyme preparations. The over-all mean of the test groups shows only 61 per cent activity by oral administration compared to 99 per cent by injection.

A similar mean of all the groups given calcium pantothenate by injection

shows an average of 96 per cent activity compared to calcium pantothenate given orally. It thus appears that calcium pantothenate is equally active

TABLE I  
*Comparative Potency of Pantothenic Acid and Coenzyme A by Two Methods of Administration*

Assay No.	Dietary supplement	Method of administration	Daily dose	Pantothenic acid equivalent*	Gain in weight	Pantothenic acid determined	
						In daily dose	Per cent of expected
1 (10 days)	Calcium pantothenate	Oral	0	0.0	7.6		
	" " "	"	25	21.2	22.1		
	" " "	"	50	42.4	31.1		
	" " "	"	100	84.9	44.5		
			mg.				
	Coenzyme Preparation 1	"	1	23.0	17.7	13.0	56
	" " " 1	"	2	46.0	29.7	39.0	85
	" " " 1	Intraperitoneal	1	23.0	22.8	23.0	100
			γ				
2 (8 days)	Calcium pantothenate	Oral	25	21.2	18.0		
	" " "	"	50	42.4	29.0		
	" " "	"	100	84.9	47.0		
	" " "	Intraperitoneal	25	21.2	19.5	24.0	113
	" " "	"	50	42.4	22.3	32.0	75
	" " "	"	100	84.9	37.7	65.0	77
3 (8 days)	" " "	Oral	0		4.0		
	" " "	"	25	21.2	19.0		
	" " "	"	50	42.4	29.0		
	" " "	"	100	84.9	47.0		
	" " "	Intraperitoneal	25	21.2	16.0	16.0	76
	" " "	"	50	42.2	36.0	60.0	141
			mg.				
	Coenzyme Preparation 2	Oral	2.61	21.2	14.0	13.0	61
	" " " 2	"	5.22	42.4	17.0	18.0	43
	" " " 2	Intraperitoneal	2.61	21.2	20.0	23.0	108
	" " " 2	"	5.22	42.4	27.0	38.0	90

\* Calculated for calcium pantothenate; as determined by microbiological assay for the coenzyme preparations.

by either route of administration and that the low activity of the coenzyme preparation when given orally results from a failure of absorption from one cause or another. Since the activity of injected coenzyme is accounted



for by its pantothenic acid content, it is unlikely that the coenzyme is utilized as such without prior hydrolysis.

#### DISCUSSION

The data show that without any previous treatment, coenzyme A, when given intraperitoneally, is utilized in place of, or as well as, free pantothenic acid. When given orally, a somewhat less complete utilization was observed, amounting to about two-thirds of the theoretical pantothenic acid value. The present data thus confirm, qualitatively and quantitatively, earlier data obtained on the pantothenic acid content in coenzyme A preparation.

The data reported, furthermore, explain observed discrepancies between the chick and microbiological tests for pantothenic acid. It has been known for some time that various materials, particularly yeast, liver, and other animal organs, yielded higher pantothenic acid values by the chick than by the microbiological assay (5, 6). As reported elsewhere, the pantothenic acid in all living cells is bound to a large extent as coenzyme. It is liberated incompletely, but to a varying extent, by autolysis and the older methods of enzymatic hydrolysis for microbiological assay. The earlier microbiological values must, therefore, be considered as distinctly too low. Chick assays more nearly reflect the true pantothenic acid content, but may be expected to be somewhat low, depending upon the availability and the amount of coenzyme present. As is the case with several of the vitamins and minerals, the question of availability to the animal must be considered as well as the total pantothenic acid content.

#### SUMMARY

Chick assays for the pantothenic acid content of coenzyme A showed that when given intraperitoneally it was as active as free pantothenic acid, and the chick assay agreed with the improved microbiological assay in which phosphatase and liver enzyme are utilized to liberate the pantothenic acid. By oral administration, however, an average of only 61 per cent of the theoretical activity was found. Pantothenic acid itself was equally active by either method of administration.

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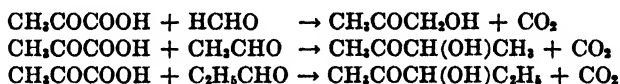
# OBSERVATIONS ON BLOOD ACETOL AFTER ADMINISTRATION OF ACETOL, METHYL ALCOHOL, AND FORMALDEHYDE TO RABBITS

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The possibility that acetol (acetylcarbinol) might be formed in methyl alcohol poisoning by the reaction of pyruvic acid and formaldehyde is suggested by the biological formation of the higher analogues, acetyl-methylcarbinol (acetoin) and acetylethylcarbinol, by the reaction of pyruvic acid with acetaldehyde and propionaldehyde respectively. The conversion of acetaldehyde to acetoin has been demonstrated *in vivo* by Stotz, Westerfeld, and Berg (11), and the conversion of propionaldehyde to acetylethylcarbinol by a carboxylase preparation from animal heart has been established by Berg and Westerfeld (1).



The biological production of formaldehyde from methyl alcohol has been detected by qualitative tests of unstated sensitivity, but the possibility of acetol formation appears not to have been investigated. Formaldehyde has been reported to occur in methyl alcohol poisoning in various organs by Pohl (9), in the vitreous humor and peritoneal fluid by Keeser (5), and in blood serum, urine, spinal fluid, and aqueous humor by Palmer and Harrop (8). Traces of formaldehyde have been detected in incubated mixtures of methyl alcohol with liver by Keeser and Vincke (6) and with brain and vitreous humor by Keeser (4). Detection of only traces of formaldehyde has in each instance been attributed to a high reactivity of formaldehyde which prevents its persistence and accumulation.

Observations have previously been made on the effect of administration of acetol to animals, but without reference to any relationship to methyl alcohol or formaldehyde metabolism. Greer, Witzemann, and Woodyatt (3) fed acetol to dogs in doses of 2 gm. per kilo, producing hematuria and hemoglobinuria, but not death. Stöhr and Müller (10) fed 1 to 2 gm. of acetol per kilo to fasting rats, causing elevation of the blood acetol concentration to 98 to 225 mg. per cent at the end of 3 hours, but with no toxic reaction discernible grossly or by any alteration of liver glycogen, alkali reserve, or urine protein content. This low toxicity found for acetol

suggests that the formation of this substance in methyl alcohol or formaldehyde poisoning would not contribute much to intoxication but might rather constitute a detoxication mechanism.

In the present study the rate at which acetol is removed from the blood stream has been determined and measurements have been made of blood acetol concentration after administration of methyl alcohol and formaldehyde to rabbits. The possible extent of conversion of methyl alcohol and formaldehyde to acetol has been estimated from the blood analysis values and the reaction rates involved.

The measurements of blood acetol in the present work have been made by a colorimetric method which is more sensitive than the titrimetric method employed by Stöhr and Müller (10). Their procedure consisted of heating blood filtrates with Folin-Wu phosphomolybdic acid reagent to give a blue color which was bleached by titration with potassium permanganate, requiring 4.4 ml. of 0.01 N oxidizing solution for 1 mg. of acetol. It was found on attempting to utilize their procedure that the end-point was difficult to recognize and that direct application of colorimetry was not satisfactory. In the new procedure, a more satisfactory color reaction for acetol was obtained with a phosphomolybdic-phosphotungstic reagent. To eliminate most of the substances of blood which can interfere in this reaction, simple low temperature vacuum distillation was employed prior to analysis.

#### *Method for Determination of Acetol in Blood*

Heparinized blood is deproteinized by mixing 1 ml. with 2 ml. of 5 per cent sulfosalicylic acid.<sup>1</sup> The mixture is centrifuged and 1 to 2 ml. of the supernatant fluid are vacuum-distilled in a simple apparatus which has been previously described (2). In this apparatus the sample is allowed to distil to dryness at room temperature, condensing at the temperature of dry ice. It is convenient to permit several samples to distil simultaneously overnight.

For colorimetric determination of acetol in the distillate, the chromogenic reagent is prepared by dissolving 26 gm. of phosphomolybdic acid and 2 gm. of phosphotungstic acid in sufficient water at room temperature to give a volume of 80 ml., and then adding 10 ml. of concentrated hydrochloric acid and 10 ml. of 85 per cent phosphoric acid, followed by removal of any insoluble material by centrifuging. Color is developed by heating together 1 ml. of blood distillate and 1 ml. of chromogenic reagent on a boiling water bath for 15 minutes and then adding immediately 2 ml. of saturated sodium

<sup>1</sup> If simultaneous distillation and measurement of formaldehyde and formic acid are desired, the sulfosalicylic acid solution should contain 0.2 M sulfuric acid, which does not influence determination of acetol.

carbonate solution. The blue color produced by acetol is measured photoelectrically with an appropriate filter.

The colorimeter calibration curve for known amounts of acetol is determined by means of a series of standard solutions containing 0 to 20  $\gamma$  of acetol<sup>2</sup> per ml. of water. The standards are allowed to react with the chromogenic reagent and are alkalized with sodium carbonate in the same manner as the blood distillates. By using a Cenco photometer with its red filter, a straight line relationship is obtained between the logarithm of the galvanometer reading and acetol concentration, as shown in Fig. 1.

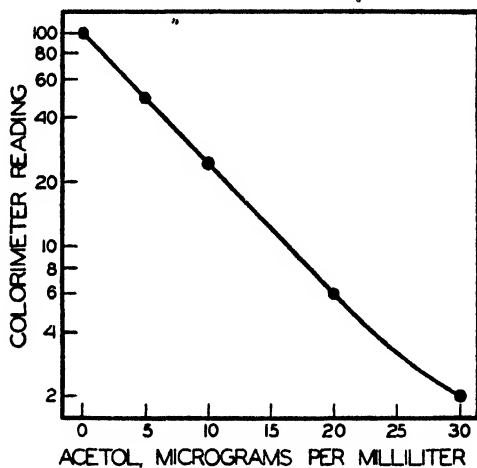


FIG. 1. Calibration curve for acetol determination

The efficiency of the recovery of acetol from blood by this method was estimated by measurements on blood to which known amounts of acetol had been added. When 10, 100, and 1000  $\gamma$  of acetol per ml. had been added, 90, 90, and 93.5 per cent respectively were recovered. In the calculation of these recovery values, a correction was made for the volumetric change resulting from the removal of the non-volatile components of blood and for the acetol blank for normal blood, which in a series of determinations on normal rabbits averaged the equivalent of 2.2  $\gamma$  of acetol per ml.<sup>3</sup>

The possibility of interference in acetol determination from methyl alcohol or its oxidation products was eliminated by ascertaining that 5 mg. of methyl alcohol, 10  $\gamma$  of formaldehyde, and 1 mg. of formic acid, singly and

<sup>2</sup> The acetol used in this investigation was prepared by the method of Levene and Walti (7).

<sup>3</sup> The blank value obtained if the distillation procedure was omitted was equivalent to 250  $\gamma$  of acetol per ml.

collectively, gave no appreciable color in the acetol analysis; nor did they influence the recovery of 10  $\gamma$  of acetol.

#### EXPERIMENTAL

*Determination of Blood Acetol Curves*—Rabbits were injected intravenously with a 10 per cent solution of acetol in water and heart blood samples were taken at intervals. The data obtained by analysis of samples

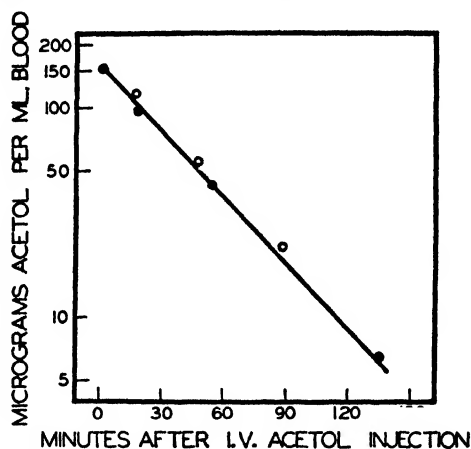


FIG. 2. Decline of acetol concentration in blood of rabbits

TABLE I

*Blood Acetol Values before and after Administration of Methyl Alcohol to Rabbits*

Amount of methyl alcohol	Time after injection	Blood acetol concentration			
	min.	$\gamma$ per ml.	$\gamma$ per ml.	$\gamma$ per ml.	$\gamma$ per ml.
2 gm. per kilo intravenously	0 (Blank)	2.5 (Rabbit A)	0.3 (Rabbit B)		
	30	1.2 " "	2.8 " "		
	90	3.0 " "	4.2 " "		
4 gm. per kilo, intraperitoneally	0 (Blank)	0.6 " C	4.0 " D	1.8 (Rabbit E)	
	210	4.0 " "	2.1 " "		
	270			7.2 " "	
	300	4.2 " "	4.5 " "		
	420	2.8 " "	2.1 " "		

from two rabbits which received 100 mg. of acetol per kilo are given in Fig. 2. It appears from these data that the blood concentration decreases exponentially with time and that the half life of acetol in the blood stream is approximately 30 minutes. The same characteristics were observed when the initial dose was reduced to approximately 40 mg. per kilo.

*Determination of Blood Acetol following Methyl Alcohol Administration—*

Methyl alcohol diluted with an equal volume of water was injected in a single sublethal dose either intravenously or intraperitoneally into five rabbits. The results of blood acetol determinations before and at various periods after injection are given in Table I.

*Determination of Blood Acetol following Formaldehyde Administration*—A solution of 5 mg. of formaldehyde per ml. of water was injected intravenously in rabbits at various constant rates for varying periods of time. The rates and times were established by previous observations to approach maximum sublethal dose conditions. In nine out of ten instances the animals survived, although some manifested severe temporary intoxication. Values found for blood acetol after administration of formaldehyde under

TABLE II  
*Blood Acetol Values during Intravenous Infusion of Formaldehyde*

Infusion rate	Duration of infusion	Blood acetol concentration	
		Experimental	Corresponding blank
<i>mg. per kg. per min.</i>	<i>min.</i>	<i>γ per ml.</i>	<i>γ per ml.</i>
1.08	62	2.4	2.4
1.26	106	1.5	1.5
1.59	50	7.2	2.1
1.64	86	7.8	1.8
1.68	159	1.8	1.8
1.77	60	1.2	3.2
1.81	101	0.9	2.1
1.92	9	0.0	3.2
1.93	44	3.9	2.6
2.65 (Died)	21	2.4	2.4
Average.....		2.9	2.3

these conditions are given in Table II along with the value for the blank blood from each rabbit for comparison.

#### DISCUSSION

The average values obtained for acetol in the blood of rabbits poisoned with methyl alcohol and formaldehyde in near lethal quantities exceed but slightly the average blank values for these animals. Furthermore, the variation in acetol concentration observed in the individual animals makes it unlikely that this small difference in the averages is significant. What this difference, if real, might mean with respect to the possible extent of acetol formation from methyl alcohol or formaldehyde may be estimated from the relative rates of disappearance of acetol, methyl alcohol, and for-

maldehyde. It is readily calculable, by utilizing the measured half life of acetol in the blood and the concentrations obtained from injection, that an increase in blood acetol of the order of 2  $\gamma$  per ml. might be produced in 60 minutes by the continuous addition of acetol to the blood stream at the rate of 40  $\gamma$  per kilo per minute. On the other hand, the theoretical potential rates of formation of acetol from sublethal amounts of methyl alcohol and formaldehyde have been calculated to be approximately 1800 and 4400  $\gamma$  per kilo per minute, respectively, based on unreported data on the rate of disappearance of methyl alcohol and formaldehyde. The questionable increase in average acetol concentration which was observed experimentally would, therefore, be equivalent to the conversion to acetol of not more than approximately 1 to 2 per cent of the methyl alcohol or formaldehyde injected.

It may be concluded that formation of acetol from methyl alcohol or formaldehyde in the rabbit does not occur to any extent which could be considered significant with respect either to an intoxication or a detoxication mechanism.

#### SUMMARY

The possibility was investigated that formaldehyde might react *in vivo* with pyruvic acid in a manner analogous to acetaldehyde and propionaldehyde. The concentration of the postulated product, acetol, was determined colorimetrically in blood samples by reaction with a mixture of phosphotungstic and phosphomolybdic acids after low temperature vacuum distillation from blood filtrate. When acetol was injected in well tolerated doses of 40 to 100 mg. per kilo intravenously in rabbits, it had a half life in the blood stream of 30 minutes and disappeared exponentially with time. When sublethal amounts of methyl alcohol or formaldehyde were administered, not more than 1 to 2 per cent, if any, was converted to acetol.

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# ON THE USE OF THE NEPHRECTOMIZED RAT FOR THE STUDY OF RAPID CHANGES IN NITROGEN METABOLISM\*

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With the exception of some recent applications of the isotope technique (1), studies concerned with endocrine influences over nitrogen metabolism have depended largely on the use of the conventional nitrogen balance technique or on measurements of tissue nitrogen. While these methods have yielded valuable information about over-all changes in nitrogen metabolism, they do not offer much opportunity to detect small or transient shifts in protein metabolism or to establish time relationships with hormone action when the latter are rapid or of brief duration. The importance of being able to measure such small and rapid changes in nitrogen metabolism is emphasized by recent studies which indicate that certain of the hormones concerned in nitrogen metabolism, and particularly the adrenal cortical hormones, act more rapidly than had been once thought (2). Evidences of adrenal cortical activity can be demonstrated as soon as 20 minutes following the injection of potent cortical hormone or after administration of adrenocorticotrophic hormone. By the conventional methods for studying nitrogen metabolism it is not possible to detect the earliest changes attendant on hormone action. Furthermore, the 12 to 24 hour urinary nitrogen excretion may reflect not only the effects of the hormone itself, which may be short lived, but the secondary homeostatic changes as well. Accurate collections of urine for less than 12 hours are difficult when small animals such as rats are used. In order to obtain more information concerning factors involved in the endocrine control of nitrogen metabolism, this investigation of use of the nephrectomized rat for the study of rapid changes in nitrogen metabolism was begun.

Since urea, the end-product of protein catabolism, is freely diffusible throughout the body water, measurement of its rate of accumulation in

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the blood of the nephrectomized animal should be a direct measure of protein metabolism, and, indeed, has been so used by several investigators (3, 4). If the rate of urea formation can be shown to be relatively constant from hour to hour, if a sufficiently sensitive method for urea determination is used, and if no significant changes in body water occur, it should be possible to detect small changes in nitrogen metabolism by this technique. These criteria were met in this investigation. It was found that the hourly rate of urea formation in the nephrectomized rat remains relatively constant for at least 22 hours. Changes in urea nitrogen formation smaller than 1.0 mg. of nitrogen per 100 gm. of body weight per hour could be detected with a high degree of accuracy. By using brief periods of observation, possible changes in body water were minimized.

### *Methods*

Male albino rats of the Sprague-Dawley strain, weighing from 200 to 250 gm., were used. The animals were fed Rockland rat chow until the time of nephrectomy. Bilateral nephrectomies through a lumbar approach were performed under pentobarbital in saline. After nephrectomy the rats were allowed free access to water but not food. Weight records were kept to detect any undue fluctuations not explicable by the overnight fast. The animals were operated on in the late afternoon and experimental procedures began the following morning, 16 hours postoperatively. The rats were kept in an air-conditioned room at 24–27° after nephrectomy. Urea nitrogen determinations were carried out in quadruplicate on 0.5 ml. of a 1:50 tungstate filtrate from 0.2 ml. of tail blood by means of the microphotometric xanthidrol method of Engel and Engel (5). Blood was collected at the time of nephrectomy and at the 16th, 19th, and 22nd hours after operation. The rats were kept under light pentobarbital anesthesia for the blood collections. Changes in urea nitrogen were expressed as mg. of urea nitrogen synthesized per 100 gm. of body weight per hour, and calculated on an assumed equal distribution of urea throughout the total body water. The total body water was taken as 63 per cent of the body weight (6).

### *Results*

Table I summarizes the results of these experiments. 113 rats, in which the rate of urea synthesis was determined during the first 16 hours after nephrectomy, had a mean rate (with standard error) of  $2.95 \pm 0.05$  mg. of N per 100 gm. of body weight per hour. 201 rats, including the above and representing the control 3 hour period for all animals studied between July, 1946, and March, 1947, synthesized urea during the 16th to 19th hours after nephrectomy at a rate of  $3.02 \pm 0.08$  mg. of N per 100 gm. of body weight

per hour. Comparison of the rates of urea synthesis during three successive periods, 0 to 16 hours, 16 to 19 hours, and 19 to 22 hours, after nephrectomy shows an equally good correspondence in twenty seven animals, the rates being respectively  $2.68 \pm 0.13$ ,  $2.80 \pm 0.19$ , and  $2.85 \pm 0.18$  mg. of N per 100 gm. of body weight per hour. From these results it is clear that under the experimental conditions herein described the rate of urea formation remains quite constant during the arbitrary periods chosen after nephrectomy. This is again demonstrated in the next seven groups of experiments recorded in Table I in which the effects of several substances

TABLE I  
*Studies of Nitrogen Metabolism in Nephrectomized Rats*

Series No.	Procedure	No. of rats	Urea N, mg. N per 100 gm. body weight per hr.*			
			0 to 16 hrs.	16 to 19 hrs.	19 to 22 hrs.	P†
1	Control	113	$2.95 \pm 0.05$			
2	"	201		$3.02 \pm 0.08$		
3	"	27	$2.68 \pm 0.13$	$2.80 \pm 0.19$	$2.85 \pm 0.18$	0.5
4	Amigen (8.4 mg. N per 100 gm.)	19	$3.11 \pm 0.10$	$2.99 \pm 0.32$	$4.35 \pm 0.19$	<0.01
5	Vuj-n (10.4 mg. N per 100 gm.)	24	$2.90 \pm 0.12$	$3.07 \pm 0.23$	$4.25 \pm 0.23$	<0.01
6	Cortin (0.4 ml. per 100 gm.)	18	$3.07 \pm 0.10$	$2.97 \pm 0.21$	$3.43 \pm 0.26$	0.2
7	Cortin (0.8 ml. per 100 gm.)	13	$3.04 \pm 0.18$	$2.86 \pm 0.22$	$4.09 \pm 0.34$	<0.01
8	Cortin (1.2 ml. per 100 gm.)	12	$3.69 \pm 0.16$	$2.76 \pm 0.21$	$3.72 \pm 0.26$	<0.01
9	Cortin (2.0 ml. per 100 gm.)	12		$2.90 \pm 0.32$	$4.03 \pm 0.34$	<0.01
10	Desoxycorticosterone acetate + Vuj-n (10.4 mg. N per 100 gm.)	7	$3.11 \pm 0.16$	$2.81 \pm 0.26$	$4.36 \pm 0.16$	<0.01

\* Standard error =  $\sqrt{\Sigma d^2/(n(n-1))}$ .

† Level of significance between the 16 to 19 hour and 19 to 22 hour periods.

affecting urea production were tested. In five of the six groups measured the 0 to 16 hour rate of urea synthesis ranged between 2.90 and 3.11 mg. of N per 100 gm. of body weight per hour, while in the sixth it was  $3.69 \pm 0.16$  mg. of N per 100 gm. of body weight per hour, a value significantly higher than the others. The only explanation for this single discrepancy in the whole series is that at the time this group was examined the room in which the animals were kept got unusually cold, thereby possibly stimulating the basal rate of nitrogen metabolism. It will be noted that this high

rate had fallen to normal after 16 hours in the air-conditioned room. In seven groups of rats, including from seven to twenty-four animals per group, the control 3 hour period of urea synthesis from the 16th to 19th hours postoperatively ranged between  $2.76 \pm 0.21$  and  $3.07 \pm 0.23$  mg. of N per 100 gm. of body weight per hour.

The data in Series 4 to 10 illustrate the application of the method to the detection of changes in nitrogen metabolism after various experimental procedures<sup>1</sup> following a 3 hour control period 16 hours after nephrectomy.

In Series 4, 8.4 mg. of N per 100 gm. of body weight as a sterile 8 per cent solution of casein hydrolysate (amigen) was injected intravenously into the external saphenous vein at the 19th hour after nephrectomy. During the 3 hour control period prior to injection, urea N was being formed at the rate of  $2.99 \pm 0.32$  mg. of N per 100 gm. of body weight per hour, while during the 3 hours after injection the hourly rate increased to  $4.35 \pm 0.19$  mg. of N per 100 gm. of body weight. This represents an increment of 1.36 mg. of N per 100 gm. per hour or 4.08 mg. of N in 3 hours; i.e., the equivalent of 48.5 per cent of the injected nitrogen was converted to urea. With another amino acid mixture, Vuj-n (Series 5) as a sterile 8 per cent solution and at a dose level of 10.4 mg. of N per 100 gm. of body weight, the urea synthesis increased from  $3.07 \pm 0.23$  to  $4.25 \pm 0.23$  mg. of N per 100 gm. of body weight per hour, a rise of 1.18 mg. of N per 100 gm. of body weight per hour, representing the conversion to urea in 3 hours of an amount of nitrogen equivalent to 34.0 per cent the Vuj-n nitrogen injected. This mixture, devised by Madden *et al.* (7), consists of the ten essential amino acids plus glycine. The changes following injection of both amino acid mixtures were highly significant statistically ( $P < 0.01$ ). Although the experimental periods chosen were 3 hours, it should be pointed out that we have shown in previous work (8) that the major conversion of amino acids to urea actually occurs during the first 2 hours after injection.

In Series 6 through 9 the effects of increasing doses of aqueous adrenal cortical extract on urea formation are described. The extract was administered subcutaneously in divided doses at the 16th and 17th hours after nephrectomy, blood being collected as before for urea determination at the time of nephrectomy, 16, 19, and 22 hours postoperatively. No changes were noted during the first 3 hour period. During the second 3 hour period a statistically significant increment ( $P < 0.01$ ) occurred with all doses tested except the smallest, 0.4 ml. per 100 gm. ( $P = 0.2$ ). The significance

<sup>1</sup> We are indebted to Dr. Warren M. Cox, Jr., of Mead Johnson and Company for the amigen, to Dr. D. F. Robertson of Merck and Company, Inc., for Vuj-n amino acid mixture, to Dr. D. J. Ingle of The Upjohn Company for aqueous adrenal cortical extract, and to Dr. E. Oppenheimer of Ciba Pharmaceutical Products, Inc., for des-oxytorticosterone acetate.

of these changes will be the subject of a later report. In Series 10, the rats received 5 mg. of desoxycorticosterone acetate for 7 days, and then the basal urea formation and that after 10.4 mg. of N per 100 gm. of body weight as Vuj-n were studied following nephrectomy. The desoxycorticosterone was found to have no apparent effect on either of these processes.

#### DISCUSSION

The data presented show that under the conditions described the rate of urea accumulation in the nephrectomized rat is relatively constant for at least 22 hours postoperatively. With a sensitive micromethod for urea determination, changes in urea production during arbitrarily chosen brief intervals can be readily detected. If the data from the 201 control 3 hour periods of urea formation (3.02 mg. of N per 100 gm. of body weight per hour with a standard deviation of  $\pm 1.01$  mg. and a standard error of  $\pm 0.08$  mg.) are used, and if a difference between the means of 2.6 times the standard error of the difference of the means as the criterion of statistical significance is accepted, it is possible to estimate the number of animals necessary to detect differences of varying magnitude in urea formation by this technique (9).<sup>2</sup> Assuming that all other conditions are kept constant as in the experiments reported, a group of nine to ten animals should suffice to reveal a change of 0.75 mg. of N per 100 gm. of body weight per hour, while a change of 0.50 mg. of N per 100 gm. of body weight per hour would be significant in a series of twenty or more rats.

The method is simple, accurate, and reliable and is applicable to a variety of problems in nitrogen metabolism. It has been used in a study of nitrogen metabolism during hemorrhage and shock (8). In this study 1 hour periods were successfully compared. The changes following injection of amino acid mixtures described in the present report suggest a possible method for assaying such mixtures for their utilization and toxicity. We have found<sup>3</sup> that when solutions of amigen became bacterially contaminated their injection resulted in the production in 3 hours of an amount of urea nitrogen representing better than 100 per cent of the injected nitrogen, indicating an actual stimulation of the catabolism of tissue protein by the injection. These findings emphasize the necessity for caution and careful controls before interpreting increases in urea after injection of test materials as either coming from the injected substances or as representing a stimulation by them of other than a non-specific protein catabolic response.

<sup>2</sup>  $N = t^2 s^2 / (\bar{X} - m)^2$ , where  $N$  = number of animals,  $t = 2.6$ ,  $s$  = standard deviation from the mean, and  $(\bar{X} - m)$  = the difference in urea N synthesis from a mean control period in mg. of N per 100 gm. per hour.

<sup>3</sup> Unpublished observations.

For best results, rigid control of the environmental conditions of the rats before and during the experimental periods is necessary. The diet must be kept constant. A series of animals examined concurrently with the above animals, but kept on a synthetic diet low in potassium, had a lower basal rate of urea production (10). Rats of a different strain, which were fed Purina fox chow supplemented with liver now being studied, also show a different basal rate from the series described here.

From the standpoint of interpretation of basic metabolic mechanisms, this method has obvious disadvantages. The operation represents a stress which has its own repercussions on nitrogen metabolism and may thereby alter the response to any given test material in an as yet unpredictable manner. In the absence of the kidneys serious metabolic derangements consequent to uremia eventually occur, making interpretation in terms of normal metabolism impossible. This is reflected in nitrogen metabolism by a constantly accelerating rate of urea production beginning about 30 hours after nephrectomy and continuing until death. This acceleration does not occur in the adrenalectomized rat (10). By using animals only during the period of constant urea production and for short periods this factor is largely obviated.

Another potential source of error is the arbitrary use of 63 per cent of the body weight to represent the total body water. This figure represents only an approximation which without actual determination in each animal may introduce an error. In any given animal, however, this error would enter equally into the control and experimental periods and hence not affect the difference. In a given series, the error would contribute to the variations in both periods. It is unlikely that important changes occur in water content in the brief periods chosen by us for these studies, since weight records have revealed no significant fluctuations.

In previous reports (8, 10-13), 75 per cent of the body weight was used as an index of total body water. Although there are some reports of values as high as this (14), the best evidence indicates that 63 per cent is closer to the true value (6, 15). For the purpose to which these studies have been put, *i.e.* the comparison of the effects of different experimental procedures on the rate of protein catabolism, the use of the higher figure for body water does not affect the results. However, if information about the absolute level of urea production is desired, a significant error would be introduced by its use.

It is important to avoid taking too large or frequent samples of blood, as it has been shown that small hemorrhages (2 per cent of body weight) may stimulate and large hemorrhages (greater than 2.5 per cent of body weight) may depress urea synthesis in the rat (8).

## SUMMARY

A study of the rate of blood urea nitrogen accumulation during the first 22 hours following bilateral nephrectomy as a measure of protein metabolism in the rat is presented. It is shown that this rate is constant enough to make possible the detection in brief periods of changes in nitrogen metabolism of the order of 1.0 mg. of N per 100 gm. of body weight per hour in groups as small as ten animals. The uses and limitations of the method for the study of nitrogen metabolism are discussed.

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# THE DISTRIBUTION OF POTASSIUM ISOTOPES IN BIOLOGICAL MATERIAL

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A suggestion that the isotopic composition of K isolated from biological material might be different from that of mineral K was offered by Loring and Druce (1), who claimed that K from the ash of potato stalks had an atomic weight of 40.5 and a radioactivity much greater (as judged by its action on photographic paper) than mineral K. These observations were repeated and extended (2), with the additional finding that dead wood did not show such a separation of the K isotopes. The above observations were not confirmed, however, by several investigators (3-6), all of whom found no difference in the atomic weight of K from plant as contrasted with mineral sources. Using a photographic method, Ernst (7) was led to conclude that K from different organs of man and animals was much more active than ordinary mineral K. Pohlmann and Netter (8) and Pohlmann (9) in an analysis of K from both plant and animal tissue, with a Geiger-Müller counter, concluded, however, that any difference in the radioactivity of such K from that of mineral K must be less than 5 per cent.

One difficulty with work on this question was the relative inaccuracy of methods of measuring and another the lack of knowledge regarding the identity of the K isotopes. The isotope  $K^{40}$  was detected by Nier (10) who suggested that it was the radioactive isotope, a view subsequently confirmed (11). An examination of the radioactivity of K isolated from tumor tissue was made by Lasnitzki (12) who found no evidence of a separation of isotopes within about 2 per cent. A similar result was found (13) for K isolated from rabbit muscle.

While the above measurements were being made on the radioactivity of K (e.g. the isotopic content of  $K^{40}$ ), another series of estimations was carried out on the distribution of the  $K^{41}$  isotope in biological material. The various K isotopes are distributed approximately as follows:  $K^{39}$  8200,  $K^{40}$  1,  $K^{41}$  580. Brewer (14) found that K from Pacific kelp is richer in  $K^{41}$  to an extent of almost 15 per cent over mineral K. Other plants were also found to differ from -2 to -3 per cent of enrichment in the heavy isotope. Measurement of the isotope ratio  $K^{39}:K^{41}$  for animal tissues (15) showed no variation except that bone marrow had a slight enrichment in  $K^{41}$  (about

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2 per cent) and the lining of the heart was lower than normal in  $K^{41}$ . No isotope separation for Li or Rb in the fossil *Cryptozoon* was found (16), but an isotope separation for K of around 2 per cent was detected. A summary of the above results is given by Brewer (17). A separation of  $K^{41}$  by tumor tissue to the extent that the ratio is 1 per cent less than in mineral K has been reported (18), while with *Valonia* and *Nitella* saps Jacques (19) finds, on the basis of analyses by Brewer, that the isotope ratio  $K^{39}:K^{41}$  is only 97.5 per cent of the normal ratio for mineral K. The accuracy of the estimation is stated as 0.21 per cent. The isotope ratios for normal rat tissues are given (20) as the same as for mineral K, except that bone, including marrow, had on the average 2.5 per cent less than the normal ratio. Further results on tumor tissue (21, 22) indicated that tumors were slightly more abundant in  $K^{39}$  (to an extent of about 1 per cent). The accuracy claimed was about 0.07 per cent. The results of Fenn, Bale, and Mullins (23) on the  $K^{40}$  isotope of ash from human sources, as measured with a Geiger-Müller counter, indicated that  $K^{40}$  is probably 2 per cent less abundant in such biological ash than is mineral K.

The more recent results of Cook (24) make it necessary to view all previous data on K separation determined with the mass spectrometer with caution. Cook finds the best relative accuracy obtainable in estimations of  $K^{41}$  to be 1 per cent, while the absolute accuracy is 2 per cent. He has determined the  $K^{39}:K^{41}$  ratio for various kelps, fossil *Cryptozoon*, and various minerals and can find no difference in the isotopic ratio compared with mineral sources. He has observed, however, that the mass spectrometer can itself cause "isotope effects" in the emission of K ions from its filament. It seems that previous results showing isotope separations may have resulted either from this error or from an overestimate of the accuracy of the method. Since the only other estimate of K isotope separation concerned a separation of  $K^{40}$  in human ash, it was decided to measure the radioactivity of K from biological materials, advantage being taken of the more recent developments in Geiger counting techniques.

#### EXPERIMENTAL

Potassium from wood was obtained from the trunks of beech trees. This wood was burned in a clay-lined stove and the resulting ash treated with water to extract the K. For bone K, the long bones of cows and horses were ashed in an iron tray and distilled water percolated over the ash for several hours. From lava rock (Vesuvius) the K was removed by bringing the rock in solution by HF in platinum, then treating with  $H_2SO_4$ , evaporating to dryness, and extracting with water. KCl, Merck, for analysis, was used as a control and treated for purification in the same manner as other KCl samples.

All K solutions were made neutral or slightly alkaline, if not already so, and evaporated to a volume of around 50 ml. Any precipitate was filtered off and  $\text{HClO}_4$  added in excess. The resulting  $\text{KClO}_4$  was filtered out upon glass filters and recrystallized from hot water two times, washed with absolute alcohol, and dried. The perchlorate was then placed in a muffle oven and decomposed to the chloride. Temperatures of around  $600^\circ$  were found necessary to insure this conversion. The resulting chloride was weighed to assure that the perchlorate was decomposed and the salt then dissolved in a small quantity of water and analytical grade  $\text{HCl}$  was added to recrystallize the chloride. The resulting salt was filtered on glass, washed with  $\text{HCl}$ , and dried. It was then heated to  $400^\circ$  and placed in a desiccator. The salt was ground in agate to a uniform powder, a carefully weighed aliquot was taken for chloride analysis by titration with  $\text{Ag}$ , and a sample ( $300 \pm 0.2$  mg.) was secured for counting in aluminum dishes. It was first thought that the most reproducible distribution of  $\text{KCl}$  in counting dishes could be obtained by melting the  $\text{KCl}$  into platinum counting dishes. Trials indicated, however, that the annealing of such melted salt was very difficult, and it proved impossible to prevent distortion of the platinum dishes and consequent counting error due to asymmetry of the dishes. Aluminum dishes with 300 mg. of uniformly powdered  $\text{KCl}$  were found to give reproducible counting characteristics, and were considerably easier to prepare. It must be emphasized that the weak link in many measurements of radioactivity is the lack of a reproducible geometry for the samples as they are placed in the registration apparatus. In our equipment, a change of position of the sample of 0.1 mm. will produce more than a 1 per cent change in the resulting count.

The apparatus used for counting is a slight variation of one previously described (25) and consisted essentially of a circular plate with six holes symmetrically distributed, each about 1 cm. from the circumference. These holes carry the counting dishes, and the whole plate is rotated automatically at constant intervals of time to bring each sample between two counting tubes placed above and below the plate. This arrangement provides a minimal sensitivity to changes in the height of the sample. A change in the distance of the sample from the counter will cause a change in the count of the sample. When samples are rather close to the counter, the rate of counting will be inversely proportional to the distance (over small variations in distance). This is the reason why changes in height of the samples will compensate each other when the results are expressed in per cent of a standard for both counting tubes, and a mean value taken. The counts coming from the two amplifier circuits registered on a pair of the twelve telephone message counters necessary to record from each of the six counting positions. A cut-off switch was added to the circuit to prevent the recording of any

counts during the time of rotation from sample to sample. The entire operation of the equipment being automatic, it was possible to compare over long periods of time the activities of six samples with only occasional readings of the counters. The equipment was provided with breaker controls which permanently shut off the apparatus in case of either A.C. or D.C. power failure.

In making measurements, three samples of cow bone KCl were placed in Positions 1, 3, and 5 and three KCl (Merck, purified as above) samples were placed in Positions 2, 4, and 6. The apparatus then was run for 48 hours and the results noted, and then each sample was advanced one position in the plate and counting was carried on for another 48 hours. Finally the samples were replaced in their original positions and counted again for 48 hours. All samples were calculated to per cent of the standard, and the mean value of the counts recorded by the top and bottom counters taken.

### *Results*

Since the accuracy of the measurement of particles from radioactive substances is inversely proportional to the square root of the number of counts obtained, it is obvious that to attain an accuracy of 1 per cent one must measure 10,000 counts, while for 0.1 per cent, 1,000,000 counts must be measured. Our original plan was to measure to 0.5 per cent (*e.g.* 40,000 counts) and as the K in the dishes gave approximately 30 net counts per minute, a considerable amount of time was required. A preliminary series of countings was undertaken to determine the effect of different amounts of KCl in the counting dishes. The results of this series are shown in Table I. It was possible to prepare KCl samples with less than 1 mg. of deviation from 300 mg. and most were exact to 0.2 mg. From Table I it can be seen that a change of weight of 1 mg. makes a difference in count of only 0.1 per cent; so that there is a negligible error introduced in this way. A second control was necessary to be sure that one could detect a decrease in the count of the order of 2 per cent when a dilution of this order of magnitude was present. For this test, analytical sodium oxalate was weighed and mixed by grinding in agate. The oxalate concentration was calculated to be 2.6 per cent of the total mixture. The reduction of count obtained amounted to 3.1 per cent, which is rather good agreement considering the difficulty of obtaining good mixing with dry crystals. The data are included in Table II.

Since enough of each biological sample was prepared to fill three aluminum dishes, and comparisons were made of each of these dishes, with analytical KCl as a standard, the reproducibility of the count of any of these dishes is of some interest, inasmuch as small geometrical errors in the counting apparatus are compensated by counting at different positions in

the sample holder. Table III indicates something of the reproducibility of two dishes containing reagent KCl as counted against a third dish containing the same substance. It is to be emphasized that these results are for durations of only about 10,000 counts, and hence the accuracy expected cannot be more than 1 per cent. The mean values are about 1.5 per cent different from the control.

The final results obtained on counts for various biological materials are summarized in Table IV. Here reagent KCl is taken as 100 per cent and the various other substances related to this value. Standard deviations

TABLE I  
*Count Obtained from KCl Samples in Relation to Their Weight*

Weight	Relative count	$\Delta$ count	$\frac{\Delta \text{ count}}{\Delta \text{ weight}}$
<i>mg.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent per mg.</i>
350	100.0		
300	94.5	5.5	0.11
250	88.4	6.1	0.12

TABLE II  
*Measurement of 2.6 Per Cent Dilution of KCl with Sodium Oxalate*

Total No. of counts measured	Inactive impurity found
	<i>per cent</i>
$4 \times 10^4$	2.73
$2 \times 10^4$	2.93
$2 \times 10^4$	2.93
$2 \times 10^4$	3.86
$2 \times 10^4$	2.95
Mean value.	3.1

for these mean values are given in the last column. It is quite difficult to estimate the accuracy of these measurements. Theoretically the accuracy of the counts should be 0.3 per cent; however, there are many additional factors which contribute towards making this estimate higher. First there is the purity of the KCl preparation being counted. Previous experiments (23) have indicated that one does not find spectroscopically demonstrable Rb in the K samples isolated from animal sources. As to the presence of the radium series in these KCl preparations, coprecipitation with  $\text{Ba}^{++}$  of one of the K samples was carried out to determine whether there was any change in the count. Such a change could not be demonstrated. A further control was the titration of the chloride with silver. This procedure could

be carried out with an accuracy of 0.2 per cent, as contrasted with an analysis for K which could only be determined within 2 per cent accuracy. Variations in background were largely compensated for by long counting intervals. KCl samples as isolated from biological material did not give a flame test for Na. In all, with regard to the error, it can be said that it has not been possible to find a separation of the order of 1 per cent for the  $K^{40}$  isotope in the various materials used.

TABLE III  
*Reproducibility of Counting with Single Sample*

Trial No.	Counts, per cent of standard sample		
	Series 1	Series 2	Another sample
1	99.7	100.3	97.3
2	98.9	98.6	98.6
3	99.3	99.3	99.6
4	99.9	97.1	98.6
5	97.4		100.2
6	96.6		98.9
7	98.6		98.9
Mean.....	98.6	98.8	98.8

TABLE IV  
*Summary of Measurements on Biological Materials*

KCl sample	Total No. of counts measured	Mean value	Standard deviation
		<i>per cent</i>	
Reagent KCl.....	$3 \times 10^5$	100.0	0.26
Lava rock.....	$3 \times 10^5$	99.9	0.75
Cow bone.....	$5 \times 10^5$	100.3	0.26
Horse bone.....	$3 \times 10^5$	99.5	1.01
Beech wood.....	$5 \times 10^5$	99.8	0.17

It seems quite clear that there has not been demonstrated previously a separation of the K isotopes which is above criticism, and further it is difficult to see why one should expect such a process in living cells. The fact that isotopes should have a mobility proportional to the square root of their masses has often been mentioned as a possibility, but in reality this holds true only for the gaseous state, while in solution one must expect that the transport of these isotopes will depend on differences in their atomic radius.

This difference between  $K^{39}$  and  $K^{40}$  is practically nil. To be sure, alkali isotopes have been separated by exchange reactions in zeolites, but it is

difficult to imagine the living cell as providing the necessary conditions for such a mechanically repetitive process as isotope separation.

We are indebted to Professor N. Bohr for supplying the numerous facilities to make this work possible, to Professor G. Hevesy for his kind interest in the work, and to Professor Noe-Nygaard for supplying the lava rock samples used.

#### SUMMARY

The  $K^{40}$  content of potassium from animal, vegetable, and mineral sources has been compared with reagent KCl. No difference in  $K^{40}$  content within  $\pm 0.5$  per cent could be demonstrated for these various materials.

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# BIOSYNTHESIS OF TRICARBOXYLIC ACIDS BY CARBON DIOXIDE FIXATION

## I. THE PREPARATION AND PROPERTIES OF OXALOSUCCINIC ACID\*

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As previously reported, the biological conversion of *d*-isocitric acid to  $\alpha$ -ketoglutaric acid and carbon dioxide occurs in two steps with oxalosuccinic acid (OSA) as an intermediate; both steps are reversible and enzyme-catalyzed (1, 2).

The conversion of isocitric acid to OSA had been postulated by Martius (3) by analogy with the biological dehydrogenation of other hydroxy acids to the corresponding keto acid. Although free OSA had never been prepared, Martius assumed that this  $\beta$ -keto acid would be so unstable that it would decarboxylate spontaneously, *i.e.* non-enzymatically, to  $\alpha$ -ketoglutaric acid and  $\text{CO}_2$  at a rapid rate. The preparation of OSA (1) made possible a better understanding of the mechanism of biological reactions involving tricarboxylic acids. Since this keto acid is indeed very unstable, its preparation and properties will be described in some detail in this paper.

As is well known, when triethyloxalosuccinic ester (OS ester) is refluxed with relatively dilute acid, it is quantitatively hydrolyzed and decarboxylated to  $\alpha$ -ketoglutaric acid (4, 5). However, if it is hydrolyzed with concentrated hydrochloric acid, at room or lower temperature, OSA is formed in good yields. The free acid can then be isolated as the barium salt after removal of unhydrolyzed ester by extraction with ether.

Both OS ester and OSA give, with ferric chloride, color reactions which have proved extremely valuable as a guide in the various steps of the preparation of the acid. Both compounds give a deep red color with ferric iron but, while the color given by the ester develops gradually and is stable once its maximum is reached, with the acid the color appears instantaneously and rapidly fades to a greenish yellow. The rapid fading is due to the decarboxylation of OSA to  $\alpha$ -ketoglutaric acid catalyzed by ferric ions.  $\alpha$ -Ketoglutaric acid gives a greenish yellow color with ferric chloride (4).

Aniline markedly accelerates the decarboxylation of OSA, as is the case with other  $\beta$ -keto acids (2, 6, 7). The decarboxylation of OSA, either by

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aniline or by certain cations, can be used for the quantitative determination of the keto acid and has proved very useful in following the hydrolysis of OS ester.

#### EXPERIMENTAL

*Hydrolysis of OS Ester*—42.0 cc. (45.7 gm.) of OS ester (equivalent to 31.6 gm. of OSA) were shaken with 126 cc. of concentrated hydrochloric acid<sup>1</sup> until all the ester went into solution, and the mixture was left at room temperature (18–20°), with occasional shaking, for 24 hours. At this time the mixture contained 11.1 gm. of OSA as determined with aniline citrate,<sup>2</sup> corresponding to 35 per cent hydrolysis. The solution was concentrated *in vacuo* at 25° (bath temperature not over 35°) to about half its original volume in order to remove ethyl alcohol and facilitate further hydrolysis of the ester. The residue was made up to the original volume (168 cc.) with concentrated hydrochloric acid and the solution was allowed to stand at

TABLE I

*Course of Hydrolysis of Oxalosuccinic Ester*

5.4 gm. of ester (equivalent to 3.74 gm. of oxalosuccinic acid) hydrolyzed with 15 cc. of concentrated HCl at 20°.

Hydrolysis time	Oxalosuccinic acid found	Hydrolysis
<i>hrs.</i>	<i>gm.</i>	<i>per cent</i>
17	1.22	32.5
42	1.76	47.0
70	1.80	48.0
94	1.70	45.0

room temperature for another 24 hours, when 15.8 gm. of OSA, corresponding to 50 per cent hydrolysis, were found. Prolongation of the hydrolysis time beyond 48 hours led to no further increase in the amount of OSA determined with aniline citrate (Table I).

*Removal of Unhydrolyzed Ester and Reaction Products Other Than OSA*—The solution was concentrated under reduced pressure, as above, to about 30 per cent of its original volume, and the residue was kept at 0° overnight, whereupon some crystalline material precipitated. This material contains no OSA or ester and has not been further identified;<sup>3</sup> it was filtered off with suction, on a sintered glass filter, and discarded. The clear filtrate was extracted once with 1.5 volumes of anhydrous ether at 0°. The ether ex-

<sup>1</sup> Analytical reagent grade, sp. gr. 1.18.

<sup>2</sup> See Table V.

<sup>3</sup> The material is sparingly soluble in water and its solutions are weakly acid. After boiling with alkali, a 2,4-dinitrophenylhydrazone, closely resembling that of  $\alpha$ -ketoglutaric acid, is obtained.

tract contained most of the unhydrolyzed (or partially hydrolyzed) ester, as indicated by ferric chloride tests, and was discarded. The aqueous layer was further concentrated under reduced pressure to about 20 cc., and the residue, a thick yellow oil containing 11.0 gm. of OSA,<sup>4</sup> was made up to 26 cc. with concentrated hydrochloric acid and allowed to stand overnight at 0°. A second crop of crystals, of the unidentified material referred to above, was filtered off and discarded. The clear filtrate (23 cc.) contained 11.0 gm. of OSA.

*Precipitation of OSA As Barium Salt*—Since it was not possible to crystallize the free OSA, it was decided to isolate it as the barium salt. This offered a possibility of separating OSA from its decarboxylation product,  $\alpha$ -ketoglutaric acid, by fractionation of the barium salts.

23 cc. of the strongly acid filtrate were chilled to  $-12^\circ$ , diluted to about 90 cc. by slowly adding ice water, and brought (with vigorous mechanical stirring) to just below pH 5.0 by the very slow addition of 26 cc. of carbonate-free 28 per cent sodium hydroxide previously chilled to  $-10^\circ$ . The temperature was never allowed to rise above  $-2^\circ$ . These precautions are essential, because OSA, while stable in concentrated hydrochloric acid, readily undergoes decarboxylation in dilute acid. The solution was cooled to  $-10^\circ$ , and 150 cc. of carbonate-free 25 per cent barium acetate of the same temperature were added with mechanical stirring; a bulky white precipitate was obtained. The mixture was now brought to pH 7.3 with 8.0 cc. of 28 per cent sodium hydroxide, and alcohol (at  $-10^\circ$ ) was added to a final concentration of about 20 per cent. After stirring for 10 to 15 minutes the barium salt was centrifuged off at 0° and the mother liquor was discarded. The mother liquor contains small amounts of OS ester as well as barium  $\alpha$ -ketoglutarate which precipitates on addition of more alcohol and can be identified as the 2,4-dinitrophenylhydrazone. The barium salt was washed successively in a refrigerated centrifuge at 0° with 25, 50, and 100 per cent ethanol and with ether, and was dried *in vacuo* over  $\text{CaCl}_2$  and paraffin at room temperature. The yield of barium salt, containing 32.2 per cent of OSA, was 25.0 gm., corresponding to 8.0 gm. of OSA, or 25 per cent of theory. Kept over calcium chloride in the ice box, the barium salt is perfectly stable.

Some analytical data obtained on this preparation are shown in Table II. On the basis of the OSA content (after correction for the loss of water on further drying of a small sample *in vacuo* over  $\text{P}_2\text{O}_5$  at room temperature) the barium salt is 70 per cent pure.<sup>5</sup> A small fraction of the impurities is

<sup>4</sup> The OSA lost after ether extraction (4.8 gm.) may consist to a large extent of partially hydrolyzed OS ester.

<sup>5</sup> A smaller batch of barium salt (2.6 gm.), prepared by the same method with the same yield, was 80 per cent pure with regard to OSA ( $\text{P}_2\text{O}_5$ -dried salt: Ba, 46.0 per cent; OSA, 38.5 per cent; free  $\alpha$ -ketoglutaric acid, 2.0 per cent).

$\alpha$ -ketoglutaric acid (5 per cent); the remainder might largely consist of the unidentified material that crystallizes on concentrating and chilling the acid hydrolysates. Ferric chloride tests indicate that the barium salt contains little, if any, ester.

A solution of OSA, from 100 mg. of the barium salt, was boiled for a few minutes to decarboxylate the acid, cooled, and treated with 2,4-dinitrophenylhydrazine in 2 N HCl. A hydrazone was obtained which after two recrystallizations from hot water (yield 7.9 mg.) melted at 220° (uncorrected). A sample of  $\alpha$ -ketoglutaric 2,4-dinitrophenylhydrazone, prepared

TABLE II  
*Analytical Data of Neutral Barium Salt of Oxalosuccinic Acid*

	Found, Ba salt dried <i>in vacuo</i> over CaCl <sub>2</sub>		Calculated for C <sub>6</sub> H <sub>6</sub> O <sub>6</sub> Ba	Purity
	Uncorrected	Corrected for 5.3 per cent H <sub>2</sub> O*		
	per cent	per cent	per cent	per cent
Ba†.....	43.5	46.0	52.3	70.0
Oxalosuccinic acid‡.....	32.2	34.0	48.3	
Total $\alpha$ -ketoglutaric acid§....	29.6	31.2	37.2	
$\alpha$ -Ketoglutaric acid  .....	4.8	5.1	0.0	

\* On further drying *in vacuo* over P<sub>2</sub>O<sub>5</sub> the salt lost 5.3 per cent weight.

† Determined by the method of King (8). Average of two duplicate estimations.

‡ Determined with aniline citrate (see Table VI).

§ Determined by the hydrazone method (9) after heat decarboxylation of the oxalosuccinate.

|| Ketoglutaric acid in excess of that calculated from the oxalosuccinate present.

and recrystallized in the same manner, melted at 219° (uncorrected). Mixed m.p., 220°.

*Stability of OSA*—OSA is much less stable than oxalacetic acid and readily undergoes decarboxylation to  $\alpha$ -ketoglutaric acid and CO<sub>2</sub> in aqueous solution. The rates of spontaneous decarboxylation of the two  $\beta$ -keto acids were determined in 0.18 M acetate buffer, pH 5.1, at 25°, with the results shown in Table III. It may be seen that OSA decarboxylates about 8 times faster than does oxalacetic acid.

*Effect of Cations*—Krebs has shown that a number of polyvalent cations catalytically accelerate the decarboxylation of oxalacetic acid (10). Table IV, where the results obtained with oxalacetic acid under identical conditions are included for comparison, shows that this also holds true for OSA.

TABLE III

*Comparison of Rates of Spontaneous (Non-Enzymatic) Decarboxylation of Oxalacetic and Oxalosuccinic Acids*

1.5 cc. of 0.3 M acetate buffer, pH 5.1, made up, with keto acid and water, to a volume of 2.5 cc. Oxalacetic acid, 19 micromoles (equivalent to 425 c.mm. of CO<sub>2</sub>); oxalosuccinic acid, 16 micromoles (equivalent to 360 c.mm. of CO<sub>2</sub>). Gas, air; temperature, 25°. Solution of keto acids tipped in from the side bulb of Warburg vessels after temperature equilibration.

Oxalacetic acid			Oxalosuccinic acid		
Time	CO <sub>2</sub> evolved	k*	Time	CO <sub>2</sub> evolved	k*
min	c.mm.	× 10 <sup>-3</sup>	min.	c.mm.	× 10 <sup>-3</sup>
15	16	1.25	5	47	1.22
30	40	1.44	10	89	1.21
45	60	1.48	15	122	1.20
60	74	1.38	20	150	1.18
120	142	1.46	25	170	1.11
			30	189	1.08
Average.....		1.40			1.17

\*  $k$  = first order velocity constant of decarboxylation =  $1/t \log (a/(a-x))$  ( $t$  = time in minutes;  $a$  = initial amount of keto acid;  $x$  = amount decarboxylated at time  $t$ ).

TABLE IV

*Effect of Cations on Decarboxylation of Oxalosuccinic Acid*

Experimental details as in Table III except that the keto acids were measured into the main space of the Warburg vessels. The metallic salts were tipped in from the side bulbs after temperature equilibration.

Salt	CO <sub>2</sub> evolved	
	Oxalacetate, 15 min.	Oxalosuccinate, 5 min.
	c.mm.	c.mm.
None.....	16	47
0.001 M CaCl <sub>2</sub> .....	17	36
0.001 " MgCl <sub>2</sub> .....	17	50
0.001 " MnCl <sub>2</sub> .....	25	41
0.001 " ZnSO <sub>4</sub> .....	34	76
0.001 " CuSO <sub>4</sub> .....	158	187
0.001 " FeSO <sub>4</sub> .....	72	110
0.001 " FeCl <sub>3</sub> .....	122	245
0.001 " Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> .....	232	255

### Methods

**Determination of OSA with Aniline Citrate**—The method of Edson (7) was used. Details of its application to the quantitative estimation of OSA, both in hydrolysates of OS ester and in the barium salt, are given in Tables V and VI.

**Ferric Chloride Tests**—The color reaction of OS ester with ferric chloride was described by Wislicenus and Waldmüller (11). When used on hydrolysates, it is best to neutralize to a weak acid reaction before adding a drop or two of concentrated  $\text{FeCl}_3$  to a dilute sample of the hydrolysate.

TABLE V

#### *Determination of Oxalosuccinic Acid in Hydrolysates of Its Triethyl Ester*

Contents of Warburg vessels, main space, 0.5 cc. of 50 per cent citric acid and 1.4 cc. of water; Side Bulb 1, 0.05 cc. of 30 per cent  $\text{NaOH}$ ; \* Side Bulb 2, 0.4 cc. of aniline citrate. Main space of Vessel a (blank), 0.05 cc. of concentrated  $\text{HCl}$ ; main space of Vessel b, 0.05 cc. of hydrolysate. Gas, air; temperature,  $25^\circ$ . Contents mixed after temperature equilibration.

Time	$\text{CO}_2$ evolved	
	Vessel a	Vessel b
min.	c.mm.	c.mm.
5		433
12	75	465
15	75	465
$\text{CO}_2$ evolution corrected for blank, c.mm.....		390
Oxalosuccinic acid in 0.05 cc. hydrolysate, mg.....		3.3
" " " 168 " " gm.....		11.1

\* For approximate neutralization of hydrolysate.

When OSA accumulates in hydrolysates, one obtains an instantaneous red color, which fades rapidly, followed by the gradual development of a stable dark red. As mentioned above, the "fading" reaction is due to OSA and the gradually developing stable reaction to OS ester. When the barium salt is used, it is best to bring it first into solution with a drop or two of dilute acid. Best results are obtained, in general, at weakly acid reaction.

**Preparation of Solutions of OSA from Its Barium Salt**—Owing to the instability of OSA, all operations are carried out at  $0^\circ$ . The barium salt is suspended in a little water, brought into solution with a few drops of 2 N  $\text{HCl}$ , and the barium precipitated with the calculated amount of 1 N  $\text{H}_2\text{SO}_4$ . The precipitate of barium sulfate is centrifuged off and discarded without washing, and the supernatant is brought to the desired pH and volume. A number of aniline citrate estimations of the OSA in solution,

carried out immediately after its preparation, indicated that on the average it corresponds to 70 per cent of the calculated amount. When more exact data are required, an aniline citrate estimation is carried out simultaneously with the experiment in which the particular OSA solution is used. Solutions of OSA are conveniently made up just before use, but, in most cases, they can be used over a period of several hours if kept at 0°.

*Preparations*—The potassium salt of triethyl oxalosuccinate was prepared by the method of Wislicenus and Waldmüller (11) and converted to the free ester as described by Neuberg and Ringer (5). Oxalacetic acid was pre-

TABLE VI

*Determination of Oxalosuccinic Acid Content of Barium Oxalosuccinate*

The solid barium salt was weighed into one side bulb of the Warburg vessels. The other side bulb contained 0.4 cc. of aniline citrate and the main space 0.5 cc. of 50 per cent citric acid plus 1.5 cc. of water. Gas, air; temperature, 25°. Contents mixed after temperature equilibration.

Time	CO <sub>2</sub> evolved			
	No Ba salt	12.7 mg. Ba salt	12.3 mg. Ba salt	12.5 mg. Ba salt
min.	c.mm.	c.mm.	c.mm.	c.mm.
6		439		
10	-11	462	436	441
15	-11	472	450	455
18	-11		450	455
20		479		
30		479		
Total CO <sub>2</sub> evolved, c.mm.....		479	450	455
CO <sub>2</sub> after correcting for blank, c.mm.....		490	461	461
Oxalosuccinic acid, mg.....		4.15	3.92	3.96
" " %.....		32.7	32.0	31.8

pared by the method of Wohl *et al.* (12); its purity, as determined with aniline citrate, was 100 per cent.

## SUMMARY

The preparation of oxalosuccinic acid, by hydrolysis of its triethyl ester with concentrated hydrochloric acid at room temperature followed by removal of unhydrolyzed ester with ether and isolation as the barium salt, is described.

In aqueous solution oxalosuccinic acid is much less stable than oxalacetic acid and readily undergoes decarboxylation to  $\alpha$ -ketoglutaric acid and carbon dioxide. At pH 5.1 (0.18 M acetate buffer) and 25°, the first order

velocity constants of decarboxylation are as follows:  $k$  oxalacetate =  $1.4 \times 10^{-3}$ ,  $k$  oxalosuccinate =  $1.17 \times 10^{-2}$ .

The decarboxylation of oxalosuccinate is markedly accelerated by some polyvalent cations (in order of effectiveness  $\text{Zn}^{++}$ ,  $\text{Fe}^{++}$ ,  $\text{Cu}^{++}$ ,  $\text{Fe}^{+++}$ ,  $\text{Al}^{+++}$ ) and by aniline.

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# BIOSYNTHESIS OF TRICARBOXYLIC ACIDS BY CARBON DIOXIDE FIXATION

## II. OXALOSUCCINIC CARBOXYLASE\*

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Although oxalosuccinic acid (OSA) is very unstable (1) and rapidly undergoes decarboxylation to  $\alpha$ -ketoglutaric acid and carbon dioxide in aqueous solution, this reaction is markedly accelerated by oxalosuccinic carboxylase, a soluble enzyme, first found in pig heart extracts (2). This enzyme, which is also present in other tissues, is thermolabile, specific for OSA, and requires manganous ions for activity.

### Results

*Specificity of Enzyme*—Because of the lability of OSA, the activity of oxalosuccinic carboxylase is best investigated at low temperatures, such as 15° or lower. The enzymatic activity of dialyzed extracts of washed acetone-dried pig heart is illustrated in Fig. 1, which also shows the thermolability and the  $Mn^{++}$  dependence of the enzyme.

The decarboxylation of oxalacetic acid (OAA) to pyruvic acid and carbon dioxide had been known for some time to be catalyzed by enzymes from various sources. Oxalacetic carboxylase was first discovered by Krampitz and Werkman (3) in lysed preparations of *Micrococcus lysodeikticus*; this enzyme was stated to require either  $Mg^{++}$  or  $Mn^{++}$  for activity. Later Evans, Vennesland, and Slotin (4) found a similar enzyme in aqueous extracts of acetone-dried pigeon liver; this enzyme functioned with  $Mn^{++}$  but not with  $Mg^{++}$ .

The question whether the decarboxylation of OSA and OAA is catalyzed by the same or by different enzymes was of great interest and was investigated in some detail. It was found early in this work that pig heart extracts, with high oxalosuccinic carboxylase activity, were inactive toward OAA (2), while pigeon liver extracts decarboxylated the two keto acids. A partially purified preparation of oxalacetic carboxylase from *Micrococcus lysodeikticus*<sup>1</sup> is free from oxalosuccinic carboxylase. These facts are il-

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<sup>1</sup> Mehler, A. H., Kornberg, A., Grisolia, S., and Ochoa, S., unpublished.



lustrated in Fig. 2. More recently it has been found that highly purified preparations of "oxalacetic carboxylase" from pigeon liver<sup>2</sup> are devoid of oxalosuccinic carboxylase activity.<sup>3</sup> It is thus clear that oxalosuccinic and oxalacetic carboxylases are distinct enzymes, each being strictly specific for its substrate. Such specificity has also been found in spectrophotometric experiments (6).

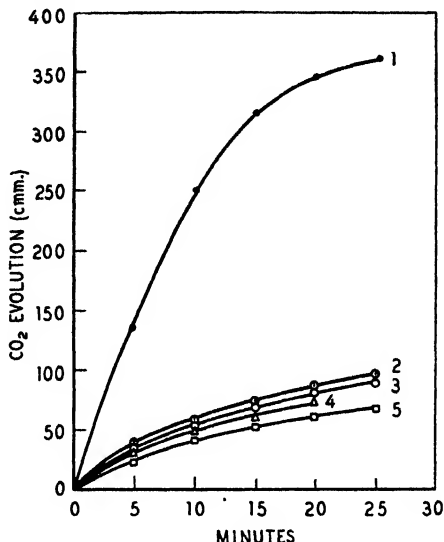


FIG. 1. Enzymatic decarboxylation of oxalosuccinic acid. 0.018 M citrate buffer, pH 5.6, and 18 micromoles of oxalosuccinate (equivalent to 405 cmm. of  $\text{CO}_2$ ), with additions as indicated, in a final volume of 2.8 cc. Enzyme, dialyzed extract of washed, acetone-dried pig heart containing about 5 mg. of protein per cc. Oxalosuccinate tipped in from side bulbs of Warburg vessels after temperature equilibration. Gas, air; temperature,  $14^\circ$ . Curve 1, 1 cc. of enzyme + 3.6 micromoles of  $\text{MnCl}_2$ ; Curve 2, no enzyme but 3.6 micromoles of  $\text{MnCl}_2$ ; Curve 3, 1 cc. of enzyme but no  $\text{MnCl}_2$ ; Curve 4, 1 cc. of heated enzyme (heated for 2 minutes at  $100^\circ$ , cooled, and coagulated protein removed by centrifugation), either with or without 3.6 micromoles of  $\text{MnCl}_2$ ; Curve 5, neither enzyme nor  $\text{MnCl}_2$ .

<sup>2</sup> This enzyme catalyzes the reversible oxidative decarboxylation of *L*-malic acid to pyruvic acid and  $\text{CO}_2$ , with triphosphopyridine nucleotide as coenzyme, as well as the decarboxylation of OAA.  $\text{Mn}^{++}$  is needed in both cases. All the evidence so far obtained indicates that the same enzyme catalyzes the two reactions; this enzyme, therefore, would not be primarily an oxalacetic carboxylase. The enzyme from *Micrococcus lysodeikticus*, on the other hand, is totally inactive toward malic acid, whether with di- or triphosphopyridine nucleotide, and is thus a true oxalacetic carboxylase. Whether the catalysis of OSA decarboxylation is an incidental activity of an enzyme, which essentially catalyzes the oxidative decarboxylation of isocitric acid to  $\alpha$ -ketoglutaric acid and  $\text{CO}_2$ , remains to be decided by purification (see the discussion on this point in Paper III (5)).

<sup>3</sup> Ochoa, S., Mehler, A. H., and Kornberg, A., unpublished.

**Reaction Products**—The enzymatic decarboxylation of OSA was allowed to proceed to completion in three Warburg vessels set up as the sample corresponding to Curve 1 in Fig. 1. The OSA content of the solution used in this experiment was simultaneously determined in another Warburg vessel with aniline citrate (1). The experimental temperature was 15°. Decarboxylation was complete in 30 minutes when 455, 423, and 440 c.mm.

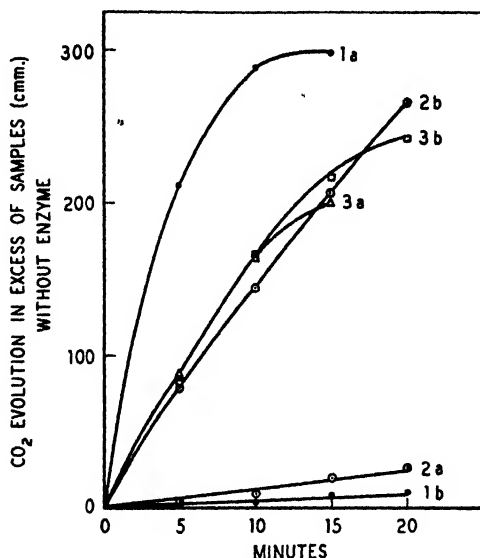


FIG. 2. Specificity of oxalosuccinic and oxalacetic carboxylase. Either 0.018 M citrate buffer, pH 5.6, and 3.6 micromoles of  $MnCl_2$ , made up with enzyme, substrate, and water, to a volume of 2.8 cc. (Curves 1a, 1b, 2a, 2b, and 3a), or 0.025 M acetate buffer, pH 5.0, and 1.0 micromole of  $MnCl_2$  made up with enzyme, substrate, and water to a volume of 2.0 cc. (Curve 3b). Curves a, substrate oxalosuccinate (18 micromoles); temperature, 16–17°; curves b, substrate oxalacetate (28 micromoles); temperature, 25°. Curves 1, 1.0 cc. of pig heart extract (4.4 mg. of protein); Curves 2, 1.0 cc. of *Micrococcus lysodeikticus* enzyme (about 1.0 mg. of protein); Curves 3, 0.5 cc. of pigeon liver extract (18.5 mg. of protein). Gas, air. Substrate tipped in from side bulbs of Warburg vessels after temperature equilibration.

of CO<sub>2</sub> were evolved in each of the three Warburg vessels, as measured from the moment OSA was tipped into the main compartment. The corresponding CO<sub>2</sub> evolution with aniline citrate was 459 c.mm. The contents of the three vessels were then mixed and deproteinized with trichloroacetic acid. Addition of 2,4-dinitrophenylhydrazine in 2.0 N HCl yielded a hydrazone which after two recrystallizations from hot water (yield 5.0 mg.) melted at 220° (uncorrected). A sample of  $\alpha$ -ketoglutaric 2,4-dinitrophenylhydrazone, prepared and recrystallized in the same manner, melted at 219° (uncorrected). Mixed m.p., 218°.

*Properties of Enzyme*—The rate of enzymatic decarboxylation of OSA is about the same at pH 5.6, 6.3, and 7.4, whereas at pH 5.0 it is very low, owing to denaturation of the enzyme. Reactions more alkaline than pH 7.4 were not investigated because of the pronounced increase of the spontaneous decarboxylation of OSA with increasing alkalinity. The data at pH 7.4 were obtained with glycylglycine buffer, all others with citrate buffer. Complete evolution of the  $\text{CO}_2$  produced by decarboxylation at pH higher than 5.6 was insured by tipping acid from the side bulb of the

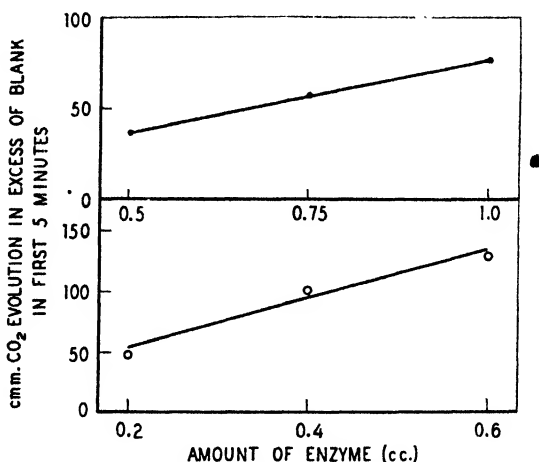


FIG. 3. Rate of enzymatic decarboxylation of oxalosuccinic acid as a function of the enzyme concentration. 0.018 M citrate buffer, pH 5.6, 3.6 micromoles of  $\text{MnCl}_2$ , and 18 micromoles of oxalosuccinate, with various amounts of pig heart extract, made up to a final volume of 2.8 cc. Other experimental details as in Fig. 1. Gas, air; temperature, upper curve,  $14.5^\circ$ , lower curve,  $17^\circ$ . (The two experiments reproduced in the figure were carried out with two different extracts.) Blank, without enzyme but with  $\text{MnCl}_2$ .

Warburg vessels, and the  $\text{CO}_2$  evolution, in the absence of enzyme, was subtracted in all cases to obtain the enzyme-catalyzed decarboxylation.

As is illustrated in Fig. 3, the initial rate of the enzymatic decarboxylation of OSA is proportional to the concentration of the enzyme.

The need of manganous ions for the activity of oxalosuccinic carboxylase has already been mentioned. It has also been previously reported (2) that  $\text{Mg}^{++}$  is ineffective at concentrations at which  $\text{Mn}^{++}$  is quite effective; this illustrated in Table I.

The increasing rate of enzymatic decarboxylation of OSA with increasing concentrations of either  $\text{Mn}^{++}$  or OSA, while the concentration of one of them is kept constant, is shown in Table II. In the case of constant OSA and increasing  $\text{Mn}^{++}$  concentration, the blank used for the whole

series was one with the highest concentration of  $Mn^{++}$ , i.e.  $1.3 \times 10^{-3} M$ . With constant  $Mn^{++}$  and increasing OSA concentration, two samples were run at each concentration of OSA, one without and one with enzyme. Optimum concentrations of  $Mn^{++}$  and OSA have not been reached in these

TABLE I

*Comparison of Effect of  $Mg^{++}$  and  $Mn^{++}$  on Activity of Oxalosuccinic Carboxylase*

0.018 M citrate buffer, pH 5.6, and 18 micromoles of oxalosuccinate, with other additions as indicated, in a final volume of 2.8 cc. Gas, air; temperature, 14°.

Additions	CO <sub>2</sub> evolved in 10 min. c.mm.
3.6 micromoles $MnCl_2$ .....	50
1 cc. heart enzyme .....	70
1 " " " + 3.6 micromoles $MgCl_2$ .....	76
1 " " " + 3.6 " $MnCl_2$ .....	284

TABLE II

*Rate of Decarboxylation of Oxalosuccinate As Function of  $Mn^{++}$  and Substrate Concentration*

0.02 M citrate buffer, pH 5.6, and 0.5 cc. of pig heart extract, with other additions as indicated, in a total volume of 2.8 cc. Gas, air; temperature, 17-18°. Oxalosuccinate tipped in from side bulb of Warburg vessels after temperature equilibration. The concentration of oxalosuccinate in the solution used for these experiments was determined with aniline citrate.

$7.6 \times 10^{-3} M$ oxalosuccinate		$1.3 \times 10^{-3} M$ $MnCl_2$	
$M \times 10^{-3} Mn^{++}$	CO <sub>2</sub> evolution* in 5 min.	$M \times 10^{-3}$ OSA	CO <sub>2</sub> evolution† in 5 min.
	c.mm.		c.mm.
0.065	27	3.04	60
0.325	57	4.55	70
0.65	87	7.60	109
1.30	110	11.40	150
		15.20	193

\* Excess over sample containing  $1.3 \times 10^{-3} M$   $Mn^{++}$  and no enzyme.

† Excess over corresponding samples without enzyme.

experiments, but it is apparent that the dissociation constants of the enzyme-Mn and the enzyme-substrate complexes are high.

Experiments on competitive inhibition of oxalosuccinic carboxylase revealed the interesting fact that, while citric acid and various keto acids do not inhibit, and *cis*-aconitic acid is only weakly inhibitory, isocitric acid is strongly inhibitory. These experiments are illustrated in Table III. Experiment 1 shows that substitution of acetate buffer of the same pH for

the citrate buffer used throughout this work makes no significant difference, indicating that citric acid does not interfere with oxalosuccinic carboxylase. Experiments 2 and 3 show that 0.01 M  $\alpha$ -ketoglutarate, oxalacetate, or pyruvate does not inhibit, while even 0.0066 M *dl*-isocitrate is strongly

TABLE III

*Competitive Inhibition of Oxalosuccinic and Oxalacetic Carboxylases*

Oxalosuccinic carboxylase, 0.02 M citrate buffer, pH 5.6, 7.2 micromoles of  $\text{MnCl}_2$ , 1.0 cc. of pig heart extract, and 21 micromoles (470 c.mm., final concentration 0.0075 M) of oxalosuccinate, in a total volume of 2.8 cc. Temperature, 12–13°. Oxalacetic carboxylase, 0.1 M acetate buffer, pH 5.2, 1.8 micromoles of  $\text{MnCl}_2$ , either *Micrococcus lysodeikticus* enzyme or purified pigeon liver enzyme, and 20 micromoles (450 c.mm., final concentration 0.01 M) of oxalacetate, in a total volume of 2.0 cc. Temperature, 25°. Gas, air. Other additions as indicated. Oxalosuccinate and oxalacetate tipped in from side bulbs of Warburg vessels after temperature equilibration.

Oxalosuccinic carboxylase			Oxalacetic carboxylase		
Experiment No.	Inhibitor	CO <sub>2</sub> evolution*  c.mm. in 5 min.	Experiment No.	Inhibitor	CO <sub>2</sub> evolution*  c.mm. in 20 min.
1		113	4†		233
		132‡		0.02 M fumarate	53
2		126		0.02 " <i>l</i> -malate	16
	0.02 M <i>dl</i> -isocitrate	0	5§		151
	0.01 " $\alpha$ -ketoglutarate	150		0.02 " fumarate	132
	0.01 " oxalacetate	116		0.02 " <i>l</i> -malate	34
	0.01 " pyruvate	139			
3		167			
	0.0066 M <i>dl</i> -isocitrate	20			
	0.013 " "	8			
	0.005 " <i>cis</i> -aconitate	127			
	0.01 " "	123			

\* Total CO<sub>2</sub> minus CO<sub>2</sub> evolved in the absence of enzyme.

† Carboxylase from *Micrococcus lysodeikticus*.

‡ 0.04 M acetate buffer, pH 5.6, instead of citrate buffer.

§ Pigeon liver enzyme.

inhibitory. It has not been ascertained whether both the *d* and *l* forms of isocitrate are inhibitory; if only the *d* enantiomorph were active, its effectiveness would be quite remarkable. It may be seen that 0.01 M *cis*-aconitate causes only about 25 per cent inhibition. It should be mentioned, in this connection, that the pig heart extracts used in this work are practically free from aconitase.

In view of the above results, it was deemed of interest to investigate the

effect of *l*-malate and fumarate on oxalacetic carboxylase. Two different enzyme preparations were used, the partially purified enzyme from *Micrococcus lysodeikticus* and a purified preparation from pigeon liver free from fumarase (7). The results (Table III, Experiments 4 and 5) show that 0.02 M *l*-malate is strongly inhibitory in both cases. There was no significant inhibition by 0.02 M fumarate with the pigeon liver enzyme, but it inhibited in the case of the bacterial enzyme; this latter result might be due to the presence of fumarase in the preparation.

Since inhibition of the enzymatic decarboxylation of OSA and OAA by isocitrate and malate, respectively, might be due in part to binding of  $Mn^{++}$  by the hydroxy acids, their effect on the decarboxylation catalyzed by  $Al^{+++}$  (1) was tested.  $Al^{+++}$  was used because, in the absence of enzyme, it is much more effective than  $Mn^{++}$  in accelerating the decarboxylation of the two  $\beta$ -keto acids. The initial rate of the  $Al^{+++}$  decarboxylation of OSA and OAA was inhibited 25 per cent by 0.02 M *dl*-isocitrate and 35 per cent by 0.02 M malate respectively. This suggests that, while some inhibition of the enzymatic decarboxylation may be due to binding of  $Mn^{++}$  by the hydroxy acids, the main mechanism is competition between hydroxy acid and keto acid for the enzyme. This is further proof of the remarkable structural specificity of these enzymes with regard to their substrates. Lwoff *et al.* (8) have recently shown that the decarboxylation of OAA by resting cell suspensions of a mutant strain of *Moraxella lwoffii* is strongly inhibited by malate.

The activity of oxalosuccinic carboxylase remains unchanged after dialysis against running tap water up to 12 hours. When precipitated by acetone at low temperatures, the activity can be recovered almost quantitatively in an aqueous solution of the precipitate. Acidification of the dialyzed extracts to pH 5.2 at 0° causes some flocculation and the almost complete disappearance of oxalosuccinic carboxylase activity from the supernatant. No activity could be recovered by redissolving the precipitate in water with the help of a few drops of dilute alkali (to pH about 7.4) either in testing this solution alone or in combination with the supernatant. Work on the purification of the enzyme is in progress.

*Distribution*—All of the enzyme extracts, with the exception of those from pigeon liver, were prepared and dialyzed as described in the section "Methods." Pigeon liver extracts were prepared as described by Evans, Vennesland, and Slotin (4) and were used without previous dialysis. For calculation of the  $Q_{CO_2}$  values, the protein content of the extracts was determined by the biuret reaction according to Robinson and Hogden (9). Table IV shows the results of tests of oxalosuccinic carboxylase activity in extracts of various tissues and, for comparison, those of corresponding tests for oxalacetic carboxylase. The latter tests were carried out either with

citrate buffer, pH 5.6, *i.e.* under the conditions of the oxalosuccinic carboxylase tests, or with acetate buffer, pH 5.0, according to the method of Evans, Vennesland, and Slotin (4).

It will be observed that oxalosuccinic carboxylase is widely distributed in animal tissues. Among the tissues tested, pig heart, pigeon breast muscle, and pig kidney exhibited the highest activity. The high activity of heart and pigeon breast muscle indicates that the enzyme is present in high concentrations in tissues having a very active, and predominantly

TABLE IV

*Distribution of Oxalosuccinic Carboxylase in Animal Tissues*

Tests with citrate buffer, 0.018 M citrate buffer, pH 5.6, and 3.6 micromoles of  $\text{MnCl}_2$  made up with enzyme, substrate, and water, to a volume of 2.8 cc. Tests with acetate buffer, 0.025 M acetate buffer, pH 5.0, and 1.0 micromoles of  $\text{MnCl}_2$  made up with enzyme, substrate, and water, to a volume of 2.0 cc. Gas, air; temperature, either 14–16° (oxalosuccinic carboxylase tests) or 25° (oxalacetic carboxylase tests). Substrates (oxalosuccinate, 18 micromoles; oxalacetate, 28 micromoles) tipped in from side bulbs of Warburg vessels after temperature equilibration.  $Q_{\text{CO}_2}$  = c.mm. of  $\text{CO}_2$  evolved (in excess of samples without enzyme) per hour and mg. of protein, calculated for the first 5 minutes after addition of substrate.

Tissue	$Q_{\text{CO}_2}$ oxalosuccinate (citrate buffer)	$Q_{\text{CO}_2}$ oxalacetate	
		Citrate buffer	Acetate buffer
Pig heart.....	840	3	0
Pigeon breast muscle.....	740	5	0
Pig kidney.....	230	14	0
" liver.....	80	20	8
Pigeon liver*.....	60	13	52
Ox brain.....	94	47	
Monkey brain†.....	45	17	

\* Enzyme prepared by the method of Evans, Vennesland, and Slotin (4).

† Acetone powder prepared in a Waring blender (kindly supplied by Dr. E. Racker).

aerobic, type of metabolism. On the other hand, as already pointed out by Evans *et al.* (4), oxalacetic carboxylase exhibits a more restricted distribution and, under the conditions chosen for extraction and testing, the enzyme appears to be absent from muscle, whether cardiac or skeletal (*cf.* (4)). When tested by the method of Evans *et al.*, with acetate buffer of pH 5.0, pigeon liver is the only tissue having a marked oxalacetic carboxylase activity. Vennesland *et al.* (10) have recently demonstrated the presence of oxalosuccinic carboxylase in parsley root preparations.

The high oxalosuccinic carboxylase activity of pig heart and pigeon breast muscle extracts and the relatively low activity of the same enzyme in

pigeon liver extracts call for some comment. Moulder, Vennesland, and Evans (11) discovered that pigeon liver extracts catalyze a dismutation between citrate and pyruvate, to yield  $\alpha$ -ketoglutarate,  $\text{CO}_2$ , and lactate, whereas pig heart or pigeon breast muscle extracts failed to catalyze such a reaction unless supplemented with a partially purified preparation of oxalacetic carboxylase from pigeon liver. This result was considered as indirect proof for the existence of an oxalosuccinic carboxylase in pigeon liver. It is clear from our results that the failure of pig heart and pigeon breast muscle extracts to catalyze the above dismutation cannot be due to lack of oxalosuccinic carboxylase. In this laboratory, we have succeeded in obtaining the Evans dismutation with pig heart extracts, supplemented with lactic dehydrogenase, by use of isocitrate rather than citrate (12).

### *Methods*

*Preparation of Extracts*—Phosphate extracts of washed, acetone-dried tissues were prepared by the method described by Straub (13) to obtain solutions of malic dehydrogenase. These extracts were dialyzed against running tap water for at least 6 hours, and a small amount of precipitate, formed on dialysis, was removed by centrifugation at low temperature. The extracts lose activity only very gradually if kept at  $4^\circ$ , and can be used for several days. Extracts prepared in this way from pig heart contain from 3 to 5 mg. of protein per cc. The heart muscle extracts are rich in oxalosuccinic carboxylase and isocitric and malic dehydrogenases; they are practically free from both lactic dehydrogenase and aconitase, which are largely removed by the preliminary washing of the minced tissue and destroyed (especially aconitase) by the acetone treatment. Their content of L-glutamic dehydrogenase is also very low.

*Manometric Tests*—Details of the manometric tests for oxalosuccinic carboxylase are given in the tables and figures. After the various reaction components except the enzyme are measured into the main space of the Warburg vessels, the vessels are placed on ice. The OSA solution is prepared at this time as described in Paper I (1) and, after measuring the enzyme into the main space, the ice-cold oxalosuccinate is placed in the side bulbs. The temperature of the bath is maintained at between  $14$ – $18^\circ$ . Oxalosuccinate is tipped in after 5 minutes of temperature equilibration. A blank is always run without enzyme to determine the rate of spontaneous decarboxylation of OSA. The pH of the buffer (5.6) must be carefully controlled, because at pH 5.2 flocculation and inactivation of the carboxylase occur.

On the basis of results obtained in a spectrophotometric study of the decarboxylation of OSA (6), a very sensitive and rapid optical test has been developed for oxalosuccinic carboxylase.



## SUMMARY

The decarboxylation of oxalosuccinic acid to  $\alpha$ -ketoglutaric acid and carbon dioxide is markedly accelerated by an enzyme present in extracts of washed, acetone-dried pig heart, pigeon breast muscle, pig kidney, and other animal tissues. Oxalosuccinic carboxylase is thermolabile and requires manganous ions for activity. The enzyme is specific for oxalosuccinic acid; it does not act on oxalacetic acid.

The enzymatic decarboxylation of oxalosuccinic acid is strongly inhibited by isocitric acid in a competitive manner; citric,  $\alpha$ -ketoglutaric, oxalacetic, and pyruvic acids do not inhibit, while *cis*-aconitic acid is weakly inhibitory.

Oxalacetic carboxylase does not act on oxalosuccinic acid. The enzymatic decarboxylation of oxalacetic acid is strongly inhibited by malic acid.

Our thanks are due to Mr. Morton C. Schneider for technical assistance. We are indebted to Mr. Alan H. Mehler for the purified oxalacetic carboxylase from *Micrococcus lysodeikticus*.

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# BIOSYNTHESIS OF TRICARBOXYLIC ACIDS BY CARBON DIOXIDE FIXATION

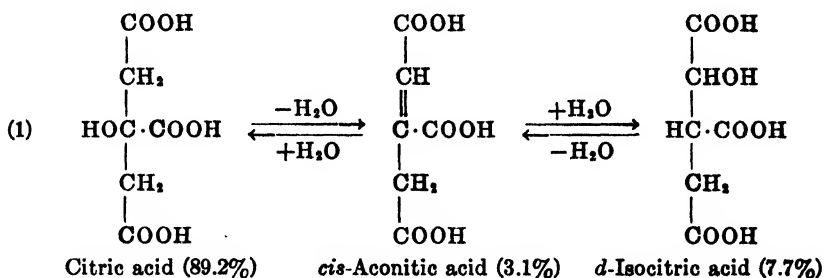
## III. ENZYMATIC MECHANISMS\*

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(Received for publication, December 29, 1947)

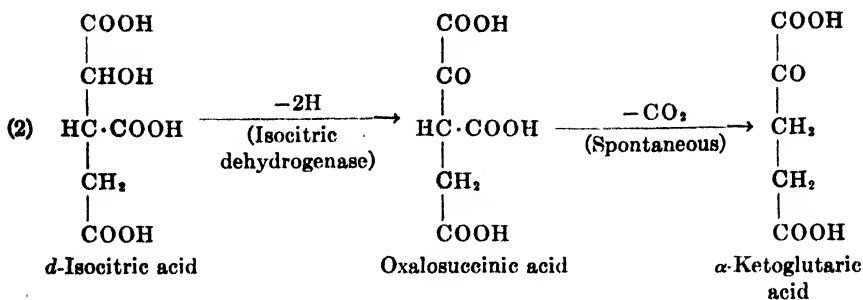
As first shown by Martius, the biological breakdown of citric acid proceeds through *d*-isocitric acid<sup>1</sup> to  $\alpha$ -ketoglutaric acid and carbon dioxide (1-3). The conversion of citric to isocitric acid is catalyzed by aconitase (4-6) and occurs according to Reaction 1, where the figures in brackets give the percentage of the three reactants at equilibrium according to Martius and Leonhardt (7).



By analogy with other hydroxy acid dehydrogenases, Martius (1) postulated that isocitric acid would first be dehydrogenated to the corresponding keto acid, *i.e.* oxalosuccinic ( $\alpha$ -keto- $\beta$ -carboxyglutaric) acid, and assumed that this  $\beta$ -keto acid would rapidly undergo non-enzymatic decarboxylation to  $\alpha$ -ketoglutaric acid and  $\text{CO}_2$  (Reaction 2).

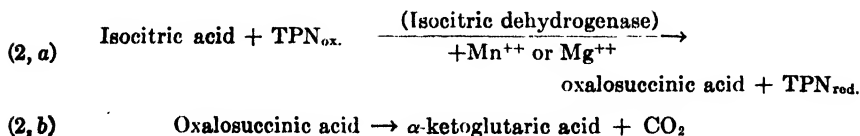
\* Aided by grants from the Rockefeller Foundation, the Penrose Fund of the American Philosophical Society, the Williams-Waterman Fund of the Research Corporation, and Hoffmann-La Roche, Inc.

<sup>1</sup> I am greatly indebted to Dr. H. B. Vickery for calling my attention to the fact that, since aqueous solutions of natural free isocitric acid are *dextrorotatory* (although the acid becomes strongly *levorotatory* in the presence of molybdenum salts), the naturally occurring isomer should be designated as *d*-isocitric acid according to the nomenclature at present in use. It was through overlooking this fact that natural isocitric acid was erroneously referred to as *l*-isocitric acid in previous publications (9, 14, 19).

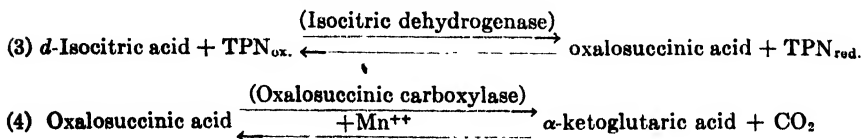


Wagner-Jauregg and Rauen (8) found that the activity of isocitric dehydrogenase was increased by crude "codehydrogenase" preparations from red blood cells which presumably contained both diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN).

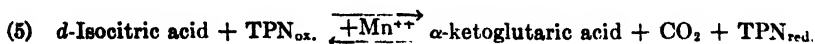
Using a spectrophotometric technique, Adler, von Euler, Günther, and Plass (3) found that isocitric dehydrogenase catalyzes the reduction of TPN, but not that of DPN, by isocitric acid, and further that the system requires either  $\text{Mg}^{++}$  or  $\text{Mn}^{++}$ , the latter being much more effective. The formulation of Martius was accepted but no evidence was obtained for the occurrence of two separate steps; *i.e.*, dehydrogenation and decarboxylation. The point of action of  $\text{Mn}^{++}$  (which was thought to be concerned with the dehydrogenation step) and the possible reversibility of the reaction also remained matters for speculation. Their results were summarized in Reactions 2, *a* and 2, *b*.



As previously reported (9), we were able to show that the biological conversion of isocitric acid to  $\alpha$ -ketoglutaric acid and  $\text{CO}_2$  is the result of two distinct, reversible enzyme-catalyzed reactions (Reactions 3 and 4).



The net result of Reactions 3 and 4 is the over-all reaction, No. 5.



The above reactions readily occur in dialyzed extracts of washed, ace-

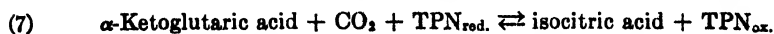
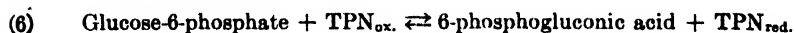
tone-dried pig heart. Reaction 5 can be followed spectrophotometrically at 340  $m\mu$ , as was done by Adler *et al.* (3), owing to the absorption of light of this wave-length by the reduced pyridine nucleotide (10). Its progress in either direction is easily followed in this way. Reaction 3 occurs in the absence of  $Mn^{++}$  and is also followed optically. Its reversibility is shown by the oxidation of  $TPN_{red.}$  on addition of synthetic oxalosuccinate (11). Reaction 4 can be demonstrated by manometric methods to run from left to right, *i.e.* in the direction of decarboxylation of oxalosuccinic acid, in the presence of enzyme and  $Mn^{++}$  (12). Since the over-all Reaction 5 is reversible, Reaction 4 must also be reversible.

The finding of Adler *et al.*, (3) that the system is strictly specific for TPN was confirmed. Not only is there no reaction with DPN, but the presence of the latter in equimolar concentration does not alter the rate of reduction of TPN, indicating that the isocitric dehydrogenase of pig heart does not significantly bind DPN. Also, in confirmation of Adler *et al.*, inorganic phosphate was found to be inhibitory.

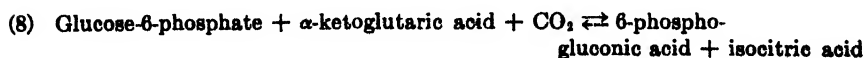
The facts just outlined suggest that Reactions 3 and 4 are catalyzed by two distinct enzymes, isocitric dehydrogenase and oxalosuccinic carboxylase respectively. However, since no separation of the two activities has yet been accomplished after partial purification by fractionation with ammonium sulfate, final decision on this point must await further purification.

It has been possible to determine the equilibrium constants of Reactions 3 and 5 from which the equilibrium constant of Reaction 4 can be calculated. The equilibrium of Reaction 5 is so far to the right that, as already observed by Adler *et al.*, if TPN is present in excess practically all the *d*-isocitric acid is converted to  $\alpha$ -ketoglutaric and  $CO_2$ . This fact makes possible a rapid and sensitive enzymatic determination of isocitric acid.

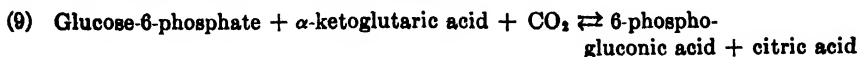
As is evident from a consideration of Reaction 5, its equilibrium can be shifted to the left, *i.e.* toward  $CO_2$  fixation, by linking it to another dehydrogenase system, of suitable oxidation-reduction potential, capable of reducing  $TPN_{ox.}$ . This has been accomplished by means of the glucose-6-phosphate dehydrogenase system of Warburg and Christian (13). The following reactions then occur.



The net result of Reactions 6 and 7 is the TPN-linked dismutation, Reaction 8.



Further shifting of the equilibrium in the direction of  $\text{CO}_2$  fixation occurs in the presence of aconitase, since over 90 per cent of the isocitric acid is then removed to form *cis*-aconitic and citric acids (*cf.* Reaction 1). Because the aconitase equilibrium is so far in favor of citric acid, the over-all reaction thus obtained can be written



The isocitric dehydrogenase-oxalosuccinic carboxylase-aconitase system can thus play an important part in the biological utilization of  $\text{CO}_2$  and may be concerned in the processes of chemosynthesis and photosynthesis. The enzymes involved are known to be widely distributed in animal, plant, and bacterial cells.

### Results

*Properties of Enzyme System*—Quartz absorption cells, of 1.0 cm. light path, were used throughout this work. The blank cell, for setting at 100 per cent transmission of light, received exactly the same additions as the experimental cell with the exception of TPN which was replaced by an equal volume of water. The final volume of the reaction mixtures was 3.0 cc. The optical measurements were carried out, at room temperature, in the Beckman spectrophotometer. The reaction was usually started by adding TPN<sub>ox.</sub> to a mixture of isocitrate and enzyme in glycine buffer, pH 7.3. There is no reaction when either isocitrate, TPN, or enzyme is omitted.

As already pointed out by Adler *et al.* (3), the affinity between isocitric acid and its dehydrogenase is extremely high; under the conditions of the optical test, Reaction 5 still proceeds at optimum rates with concentrations of *d*-isocitrate as low as  $1.2 \times 10^{-5}$  M. Since only 50 per cent of the synthetic *dl*-isocitric acid used throughout this work was found to react with TPN in the presence of the dehydrogenase, the concentrations of isocitrate given in Table I and Figs. 1 to 5 are one-half of the actual concentration used. Martius (1) has shown that levorotatory (in the presence of molybdenum salts; see foot-note 1) isocitric acid is formed by the action of aconitase on citric acid; hence *d*-isocitric acid is the natural form.

The heart extracts used in this work are practically free from aconitase; so that, at the dilutions obtaining in the optical test, there is no reaction with either citric or *cis*-aconitic acid, unless aconitase is added. This is illustrated in Fig. 1. These tests were carried out in the presence of added  $\text{Mn}^{++}$ ; *i.e.*, under conditions in which Reaction 5 is obtained. Curve 1 shows the course of the reaction with isocitric acid, Curves 2 and 3 with *cis*-aconitic and citric acids respectively; these two compounds react only after addition of aconitase at the time indicated by the arrow.

The amount of heart extract used for each test in the experiments of Fig. 1 (0.05 cc.) was much higher than usual, in order to insure an excess of isocitric dehydrogenase. For most 0.01 to 0.02 cc. is used. The heart extracts contain on the average 3 to 5 mg. of protein per cc.; so that 40 to 80  $\gamma$  of protein are generally used in a test. Salt fractionation of the extract yielded preparations of some 4 times higher specific activity.

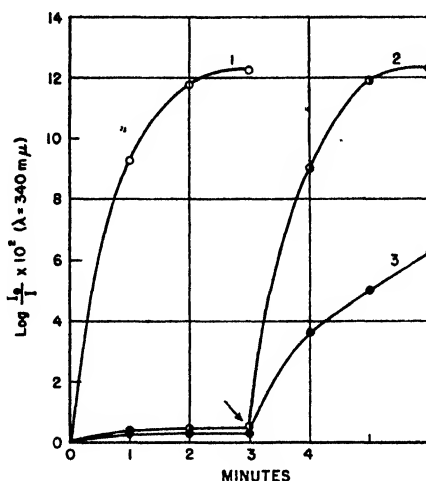


FIG. 1. Optical tests with isocitric dehydrogenase and aconitase. Reduction of TPN<sub>ox</sub>. determined spectrophotometrically at wave-length 340 m $\mu$ . Quartz cells;  $d = 1.0$  cm. 0.3 cc. of 0.1 M glycine buffer, pH 7.2, 0.05 cc. of heart extract, and 1.8 micromoles of MnCl<sub>2</sub>, with other additions as indicated, made up with water to a final volume of 3.0 cc. Temperature, 21°. 0.0662 micromole of TPN<sub>ox</sub>. added at zero time. Curve 1, with 0.126 micromole of *d*-isocitrate. (Synthetic *dl*-isocitrate was used throughout in twice the amounts given for *d*-isocitrate. The *l* isomer is biologically inert.) Curve 2, with 1.26 micromoles of *cis*-aconitate. Curve 3, with 1.26 micromoles of citrate. Aconitase (see "Methods") added at the time indicated by the arrow; Curve 2, 0.02 cc.; Curve 3, 0.05 cc. The aconitase fraction used contained 30 mg. of protein per cc.

*Isocitric Dehydrogenase*—Spectrophotometric evidence for Reaction 3 can be obtained when no Mn<sup>++</sup> is added and is given in Fig. 2. It will be seen that, with 0.0744 micromole of TPN<sub>ox</sub>. and 0.109 micromole of *d*-isocitrate, the reduction of TPN, which is rapid at first, gradually approaches an equilibrium which is reached in about 15 minutes, when only about three-fourths of the nucleotide is reduced. Addition of synthetic oxalosuccinate at this point (Arrow 2) brings about rapid reversion of the reaction toward a new equilibrium which can again be displaced in the opposite direction by increasing the isocitrate concentration (Arrow 4). At Arrow 3, addition of Mn<sup>++</sup> is followed by a rapid reduction of the TPN,

proceeding almost to completion. This indicates that Reaction 4 is now also in operation. The oxalosuccinate is decarboxylated and the equilibrium obtained corresponds to that of the over-all Reaction 5, which lies far to the right.

*Over-All Reaction*—The occurrence of Reaction 5 from left to right was established, as already mentioned, through the fundamental work of

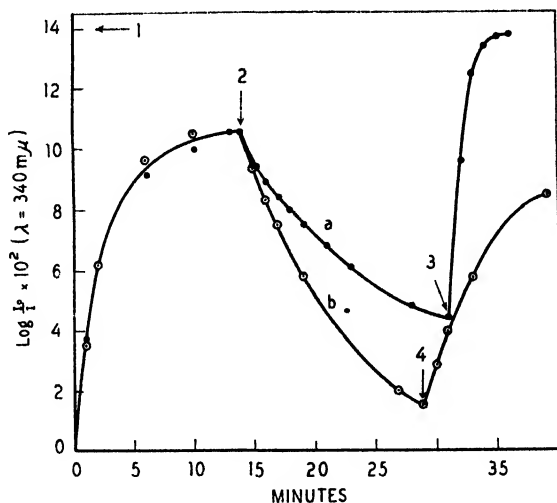


FIG. 2. Reversibility of the reaction catalyzed by isocitric dehydrogenase (Reaction 3). Reduction of  $\text{TPN}_{\text{ox}}$  and oxidation of  $\text{TPN}_{\text{red}}$  determined spectrophotometrically at wave-length  $340 \text{ m}\mu$ . Quartz cells,  $d = 1 \text{ cm}$ .  $0.3 \text{ cc}$ . of  $0.1 \text{ M}$  glycine buffer,  $\text{pH } 7.2$ ,  $0.01 \text{ cc}$ . of heart extract, and  $0.109 \text{ micromole}$  of  $d$ -isocitrate, with other additions as indicated, made up with water to a final volume of  $3.0 \text{ cc}$ . Temperature,  $23^\circ$ .  $0.0744 \text{ micromole}$  of  $\text{TPN}_{\text{ox}}$  added at zero time. Arrow 1 indicates the optical density that would be obtained if all of the added TPN were reduced when the volume is  $3.0 \text{ cc}$ . At Arrow 2, either  $0.495 \text{ micromole}$  (Curve a) or  $0.99 \text{ micromole}$  (Curve b) of oxalosuccinate was added. At Arrow 3, addition of  $1.8 \text{ micromoles}$  of  $\text{MnCl}_2$ . At Arrow 4, addition of  $0.545 \text{ micromole}$  of  $d$ -isocitrate. Curves a and b represent separate experiments.

Martius and Adler *et al.* The possibility that this reaction might be reversible was suggested by the observation that the oxidative removal of citric acid by heart homogenates was markedly diminished in the presence of arsenite, which prevents the further oxidation of the  $\alpha$ -ketoglutarate formed, so that this keto acid accumulates. When the system was inhibited by malonate instead of arsenite, citric acid was converted to succinic acid +  $\text{CO}_2$  with uptake of 1 mole of oxygen, and its removal was about 25 per cent faster than in the presence of arsenite ((14), Table I). It was subsequently found in spectrophotometric experiments that  $\alpha$ -ketoglutaric

acid definitely inhibited the reduction of TPN by isocitric acid in heart extracts. Thus, with  $8.5 \times 10^{-5}$  M isocitric acid the reaction rate was inhibited 26 per cent by  $2.3 \times 10^{-3}$  M and 41 per cent by  $4.6 \times 10^{-3}$  M  $\alpha$ -ketoglutaric acid.

The reversibility of Reaction 5 can be readily shown spectrophotometrically. On addition of  $\alpha$ -ketoglutarate to a reaction mixture containing heart extract, reduced TPN ( $\text{TPN}_{\text{red.}}$ ),  $\text{Mn}^{++}$ , and  $\text{CO}_2$ ,  $\text{TPN}_{\text{red.}}$  is oxidized, indicating progress of the reaction from right to left. The main precaution to be observed in this type of experiment is that, owing to the significant absorption by  $\alpha$ -ketoglutaric acid at  $340 \text{ m}\mu$ , the measurement of the keto acid solution into each control and experimental absorption cell should be made with the greatest possible accuracy.

Fig. 3 illustrates the effect of different concentrations of  $\alpha$ -ketoglutarate, at a constant  $\text{CO}_2$  concentration, on the extent and rate of oxidation of  $\text{TPN}_{\text{red.}}$ . On mixing isocitrate,  $\text{TPN}_{\text{ox.}}$ ,  $\text{Mn}^{++}$ , and heart enzyme, in the presence of  $\text{CO}_2$ -containing bicarbonate and glycine buffer, the pyridine nucleotide is rapidly reduced. Since at this point the total volume of 3.0 cc. has not yet been reached, the optical density of the solution is a little higher than that corresponding to reduction of all of the TPN present when in a volume of 3.0 cc. (indicated by Arrow 1). Addition of  $\alpha$ -ketoglutarate is indicated by Arrow 2. The sensitive dependence of Reaction 5 on the concentration of  $\text{CO}_2$  is illustrated by the effect of passing nitrogen through the reaction mixture to expel some  $\text{CO}_2$ . This is immediately followed by reduction of TPN and readjustment of equilibrium (Fig. 3, Curve c, Arrows 3 and 4). Fig. 4 illustrates the effect of different concentrations of  $\text{CO}_2$  on the reversal of Reaction 5. The slow oxidation of TPN that occurs on addition of  $\alpha$ -ketoglutarate without previous addition of bicarbonate- $\text{CO}_2$  is undoubtedly due to the presence of some  $\text{CO}_2$ , partly because no special precautions were taken to use  $\text{CO}_2$ -free solutions and partly because of  $\text{CO}_2$  formed from isocitric acid during the preliminary reduction of TPN. It may be seen that, for a given concentration of  $\alpha$ -ketoglutarate, the rate and extent of oxidation of  $\text{TPN}_{\text{red.}}$  are markedly dependent on the concentration of  $\text{CO}_2$ .

The need of  $\text{Mn}^{++}$  for reversal of Reaction 5 is illustrated in Fig. 5. Curve *a* (Arrow 2), given for comparison, represents the course of Reaction 5 from the beginning (since  $\text{Mn}^{++}$  was present from the beginning). Curve *b*, in the absence of  $\text{Mn}^{++}$ , represents the course of Reaction 3. On addition of  $\alpha$ -ketoglutarate, at Arrow 3, only a very slow oxidation of  $\text{TPN}_{\text{red.}}$  occurs, probably owing to the presence of traces of  $\text{Mn}^{++}$  or  $\text{Mg}^{++}$  in the reaction mixture. However, on addition of  $\text{Mn}^{++}$  (at Arrow 4) the rate of oxidation of the reduced nucleotide is significantly increased, and the reaction tends to reach equilibrium at the same  $\text{TPN}_{\text{ox.}}:\text{TPN}_{\text{red.}}$  ratio as in the control Curve *a*.



Similar results to those so far described in this section are obtained if  $\text{TPN}_{\text{ox}}$  is reduced by the glucose-6-phosphate dehydrogenase system

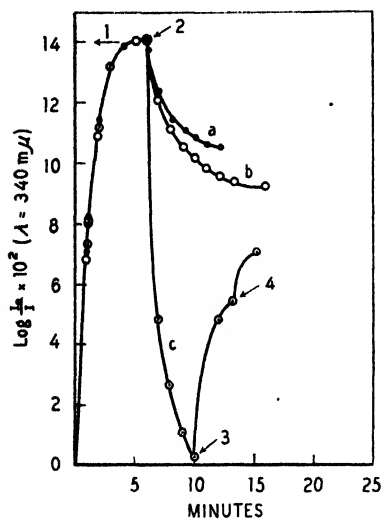


FIG. 3

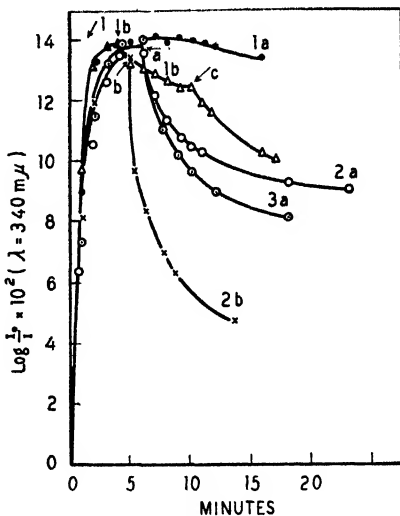


FIG. 4

FIG. 3. Reversibility of the over-all Reaction 5. Spectrophotometric determinations as in Figs. 1 and 2. 0.3 cc. of 0.1 M glycine buffer, pH 7.2, 0.3 cc. of 0.1 M  $\text{NaHCO}_3$  previously saturated with 100 per cent  $\text{CO}_2$ , 0.01 cc. of heart extract, 1.8 micromoles of  $\text{MnCl}_2$ , and 0.109 micromole of *d*-isocitrate, with other additions as indicated, made up with water to a final volume of 3.0 cc. Temperature, 22–24°. 0.0744 micromole of  $\text{TPN}_{\text{ox}}$  added at zero time. Arrow 1 as in Fig. 2. At Arrow 2, addition of  $\alpha$ -ketoglutarate; Curve a, 3.42 micromoles; Curve b, 6.84 micromoles; Curve c, 41.2 micromoles. At Arrows 3 and 4, a fine stream of nitrogen was blown through the reaction mixture for 1 minute to expel  $\text{CO}_2$ . Curves a, b, and c represent separate experiments.

FIG. 4. Effect of varying concentrations of carbon dioxide on Reaction 5. Spectrophotometric determinations as in Figs. 1 and 2. 0.3 cc. of 0.1 M glycine buffer, pH 7.2, 0.01 cc. of heart extract, 1.8 micromoles of  $\text{MnCl}_2$ , and 0.109 micromole of *d*-isocitrate, with other additions as indicated, made up with water to a final volume of 3.0 cc. Temperature, 22–24°. 0.0744 micromole of  $\text{TPN}_{\text{ox}}$  added at zero time. Arrow 1 as in Fig. 2. At Arrow a, addition of 13.7 micromoles of  $\alpha$ -ketoglutarate; Curve 1a, no  $\text{NaHCO}_3\text{-CO}_2$  present; Curve 2a, 0.15 cc. of 0.1 M  $\text{NaHCO}_3\text{-CO}_2$  (0.1 M solution of  $\text{NaHCO}_3$  previously saturated with 100 per cent  $\text{CO}_2$  at room temperature) present in the reaction mixture from the beginning; Curve 3a, 0.3 cc. of 0.1 M  $\text{NaHCO}_3\text{-CO}_2$  present from the beginning. At Arrow b, addition of 34.3 micromoles of  $\alpha$ -ketoglutarate; Curve 1b, no  $\text{NaHCO}_3\text{-CO}_2$  present; Curve 2b, 0.3 cc. of 0.1 M  $\text{NaHCO}_3\text{-CO}_2$  present from the beginning. At Arrow c, addition of 0.1 cc. of 0.1 M  $\text{NaHCO}_3\text{-CO}_2$ . Curves 1a, 2a, 3a, 1b, and 2b represent separate experiments.

(Reaction 6). This procedure has the advantage that the enzyme system used for the preliminary reduction of  $\text{TPN}_{\text{ox}}$  is different from the one used

to oxidize  $\text{TPN}_{\text{red}}$ . and, once TPN is reduced, addition of  $\text{Mn}^{++}$ ,  $\alpha$ -ketoglutarate, and bicarbonate- $\text{CO}_2$  is without effect until the heart extract, containing the isocitric dehydrogenase system, is added. Thus, it serves as a control of the experiments previously discussed in this section. This type of experiment is illustrated in Fig. 6. At time zero,  $\text{TPN}_{\text{ox}}$ . was added to a mixture of glucose-6-phosphate and glucose phosphate dehydrogenase (*Zwischenferment*) in glycine buffer of pH 7.5, and the nucleotide was

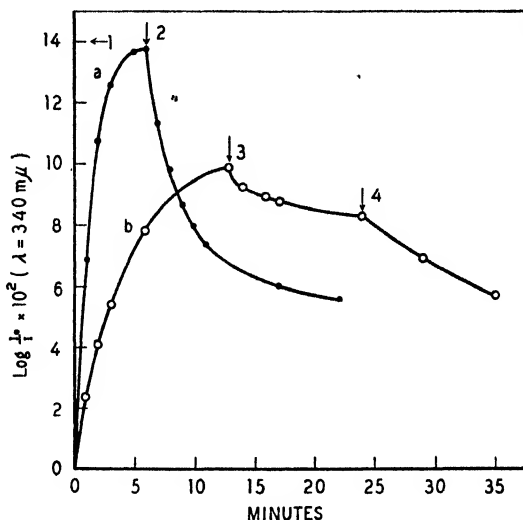


Fig. 5. Necessity of manganous ions for reversal of Reaction 5. Spectrophotometric determinations as in Figs. 1 and 2. 0.3 cc. of 0.1 M glycine buffer, pH 7.2, 0.3 cc. of 0.1 M  $\text{NaHCO}_3$  previously saturated with 100 per cent  $\text{CO}_2$ , 0.01 cc. of heart extract, and 0.109 micromole of *d*-isocitrate, with other additions as indicated, made up with water to a final volume of 3.0 cc. Temperature, 22–23°. 0.0744 micromole of  $\text{TPN}_{\text{ox}}$ . added at zero time. Arrow 1 as in Fig. 2. Curve *a*, 1.8 micromoles of  $\text{MnCl}_2$  present from the beginning. Curve *b*, no  $\text{MnCl}_2$  present at the beginning. At Arrows 2 and 3, addition of 27.4 micromoles of  $\alpha$ -ketoglutarate. At Arrow 4, addition of 1.8 micromoles of  $\text{MnCl}_2$ .

rapidly reduced as indicated by the increase in optical density. That the optical density reached is higher than that corresponding to complete reduction of the nucleotide present when in a volume of 3.0 cc. is again due to the fact that the volume of the reaction mixtures at this time is less than 3.0 cc. pending addition of further reactants. It will be observed that on addition, at Arrow 2, of  $\alpha$ -ketoglutarate,  $\text{MnCl}_2$ , and  $\text{CO}_2$ -saturated sodium bicarbonate there is an immediate drop of the optical density due to dilution of the reaction mixture. There is no other change, however, until, at Arrow 3, heart extract is added, when a rapid oxidation of TPN sets in

(Curve *a*) until a balance is reached when the reduction of  $\text{TPN}_{\text{ox.}}$  by Reaction 6 is compensated by the oxidation of  $\text{TPN}_{\text{red.}}$  by Reaction 7. The over-all reaction occurring under this condition is Reaction 8, which will be discussed in more detail in a following section. Curve *b* was obtained

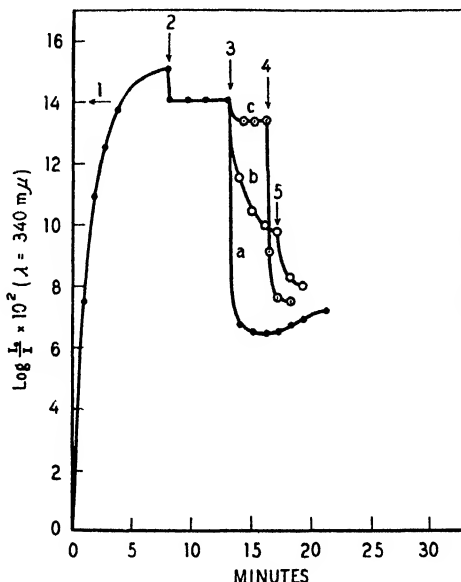


FIG. 6. Demonstration of the reversibility of Reaction 5 with the glucose-6-phosphate dehydrogenase system for the preliminary reduction of TPN. Spectrophotometric determinations as in Figs. 1 and 2. 0.3 cc. of 0.1 M glycine buffer, pH 7.5, 0.2 cc. of glucose phosphate dehydrogenase (5 mg. per cc.), and 3.23 micromoles of glucose-6-phosphate, with other additions as indicated, made up with water to a final volume of 3.0 cc. Temperature, 22–23°. 0.0744 micromole of  $\text{TPN}_{\text{ox.}}$  added at zero time. Arrow 1, as in Fig. 2. At Arrow 2, addition of 0.3 cc. of 0.1 M  $\text{NaHCO}_3$  previously saturated with 100 per cent  $\text{CO}_2$ , 1.8 micromoles of  $\text{MnCl}_2$ , and 34.3 micromoles of  $\alpha$ -ketoglutarate (Curve *a*) (decrease of optical density at this point is caused by the dilution of the reaction mixture to almost its final volume of 3.0 cc.). At Arrow 3, addition of 0.02 cc. of heart extract. Curve *b*, no  $\text{MnCl}_2$  added at Arrow 2; 1.8 micromoles of  $\text{MnCl}_2$  added at Arrow 5. Curve *c*, no  $\alpha$ -ketoglutarate added at Arrow 2; addition of 34.3 micromoles of  $\alpha$ -ketoglutarate at Arrow 4.

on addition of heart extract, at Arrow 3, to a reaction mixture to which no  $\text{Mn}^{++}$  was added, at Arrow 2. Even though there is a marked drop of optical density, it is not as rapid as that of Curve *a*, indicating that the  $\text{Mn}^{++}$  concentration is limiting. In fact, addition of  $\text{Mn}^{++}$ , at Arrow 5, markedly speeds up the reaction. This again shows that  $\text{Mn}^{++}$  is needed for reversal of Reaction 5. The fact that, in these experiments, the reaction occurs without  $\text{Mn}^{++}$  addition is probably due to the presence of  $\text{Mn}^{++}$

(and  $\text{Mg}^{++}$ ) in the crude preparation of *Zwischenferment*. Curve *c* corresponds to a sample which had not received  $\alpha$ -ketoglutarate at Arrow 2. The drop of optical density on addition of heart extract at Arrow 3 is again due to dilution of the reaction mixture; then the absorption remains stationary until, at the time indicated by Arrow 4,  $\alpha$ -ketoglutarate is added.

The reversibility of Reaction 5 has been recently confirmed by Grisolia and Vennesland using  $\text{C}^{14}\text{O}_2$  (15). The reaction has also been found to occur in parsley root preparations (16).

*Equilibrium Constants*—The equilibrium constant of Reaction 5 can be easily determined by the spectrophotometric method, since, within certain limits, if the initial concentration of reactants is known, the concentrations at equilibrium will be determined by the ratio  $\text{TPN}_{\text{ox.}}:\text{TPN}_{\text{red.}}$ .

The measurements were carried out as follows: To the absorption cells were added isocitrate,  $\alpha$ -ketoglutarate, 0.02 M  $\text{NaHCO}_3$  (equilibrated with the desired mixture of nitrogen and carbon dioxide),  $\text{MnCl}_2$ , heart extract, and water to make a volume of 2.9 cc., and the solutions were equilibrated with the  $\text{N}_2\text{-CO}_2$  gas mixture which was bubbled through the reaction mixtures in a fine stream for 3 minutes. After a zero time reading of the optical density was taken, 0.1 cc. of water was added to the blank and 0.1 cc. of TPN to the experimental cell; the reaction mixtures were again gassed for 2 minutes and the cells were closed with their covers, a little vaseline being used on the lids to insure tight closure. Readings were then taken until the optical density remained constant, indicating attainment of equilibrium; with the amount of heart extract used this occurred within 3 to 4 minutes. The measurements were carried out at room temperature ( $21\text{--}23^\circ$ ), no special precautions being taken to insure temperature constancy in the absorption cells. The concentrations of  $\text{CO}_2$  and bicarbonate in each experiment were so chosen that, from the Henderson-Hasselbalch equation, pH 6.87 should be obtained assuming  $\text{pK}_1 = 6.14$ . The pH of the reaction mixtures was always determined with the glass electrode at the end of each run and varied between pH 6.8 and 7.1.

The concentrations of *d*-isocitric acid in the solutions used for these experiments were previously determined enzymatically as described under "Methods." Since the concentrations of  $\alpha$ -ketoglutarate and  $\text{CO}_2$  are very large by comparison with those of TPN and isocitric acid, their variation in a given experiment can be neglected; hence they have been assumed to be constant in the calculation of the individual equilibrium constants. The value used for the  $\text{CO}_2$  concentration was the one calculated from the absorption coefficient ( $\alpha$ ) of  $\text{CO}_2$  by water, at the experimental temperature and  $\text{CO}_2$  partial pressure corrected for the water vapor pressure. The average room temperature of  $22^\circ$  was used in this calculation. This value, expressed as  $(\text{H}_2\text{CO}_3)$ , gives the total concentration in solution of free  $\text{CO}_2$ .

as well as of undissociated  $\text{H}_2\text{CO}_3$ . Since the equilibrium of Reaction 4 is probably dependent on the concentration of free  $\text{CO}_2$  in solution, the values for the constants obtained from  $(\text{H}_2\text{CO}_3)$  must be considered to contain the hydration equilibrium constant of  $\text{CO}_2$ .

The results of a series of measurements, in which the concentrations of  $\text{CO}_2$  and  $\alpha$ -ketoglutarate were varied within wide limits, are given in Table I. The average value for the equilibrium constant ( $K_0$ ) of the over-all

TABLE I

*Equilibrium Constant of Over-All Reaction d-Isocitric Acid + TPN<sub>ox.</sub>  $\rightleftharpoons$   $\alpha$ -Ketoglutaric Acid +  $\text{CO}_2$  + TPN<sub>red.</sub> (Reaction 5)*

0.02 cc. of heart extract, 1.8 micromoles of  $\text{MnCl}_2$ , and various amounts of 0.2 M  $\text{NaHCO}_3$  made up to a volume of 3.0 cc. with additions as indicated. Gas,  $\text{N}_2$ , with various concentrations of  $\text{CO}_2$ ; temperature, 21–23°; pH of reaction mixtures at the end of the experiment 6.8 to 7.1. Quartz cells;  $d = 1.0$  cm.

Ex- peri- ment No.	Con- cen- tra- tion of $\text{CO}_2$ in gas mixture	$(\text{NaHCO}_3)$  moles $\times 10^{-4}$ per cc.	Initial concentration of reactants, moles $\times 10^{-4}$ per cc.				Concentration of reactants at equilibrium, moles $\times 10^{-4}$ per cc.			K  $\times 10^{-4}$
			TPN <sub>ox.</sub>	d-Isoci- trate	$\alpha$ -Keto- glu- tarate	$(\text{H}_2\text{CO}_3)$	d-Isoci- trate	TPN <sub>red.</sub>	TPN <sub>ox.</sub>	
1	5.0	100	0.2392	0.2724	114.0	18.6	0.1662	0.1062	0.1330	0.98
2	5.0	100	0.2392	0.5448	114.0	18.6	0.3798	0.1650	0.0742	0.81
3	5.0	100	0.2392	0.2724	45.6	18.6	0.1464	0.1260	0.1132	1.55
4	5.0	100	0.2392	0.2724	45.6	18.6	0.1514	0.1210	0.1182	1.75
5	5.0	100	0.2392	0.2724	22.8	18.6	0.1146	0.1578	0.0814	1.39
6	10.0	200	0.2392	0.2724	91.2	37.3	0.1997	0.0727	0.1665	1.34
7	21.0	420	0.2392	0.2724	22.8	78.0	0.1751	0.0973	0.1419	1.43
8	32.0	640	0.2392	0.2724	22.8	118.0	0.1848	0.0876	0.1516	1.19
9	47.0	940	0.2392	0.5448	45.6	174.0	0.4873	0.0575	0.1817	1.94
10	2.8	56	0.2392	0.2724	114.0	10.6	0.1314	0.1410	0.0982	0.76
Average.....										1.32

Reaction 5, at the pH and temperature given, was

$$K_0 = \frac{(d\text{-Isocitrate}) (\text{TPN}_{\text{ox.}})}{(\alpha\text{-Ketoglutarate}) (\text{H}_2\text{CO}_3) (\text{TPN}_{\text{red.}})} = 1.3 \times 10^{-4}$$

The rather large variations between individual values may be due, in part, to the variations in temperature between individual experiments. It may be pointed out that Reaction 5 should more properly be written



The equilibrium constant of Reaction 3 can be determined in the same manner. However, the instability of oxalosuccinic acid makes an accurate

estimation of this constant rather difficult, and it seems desirable to postpone such measurements until purer preparations of isocitric dehydrogenase become available. An approximate estimate has, however, been obtained from data such as those used in Fig. 2. The approximate value of  $K_i = ((d\text{-isocitrate}) (\text{TPN}_{\text{ox}})/(\text{oxalosuccinate}) (\text{TPN}_{\text{red}}))$  was about 0.3 at pH 7.2 and 23°.

TABLE II

*Enzymatic Identification of d-Isocitric Acid Formed by Reaction 8*

1.0 cc. of heart extract and 2.0 cc. of glucose phosphate dehydrogenase (5 mg. per cc.) made up to 16.0 with additions including 64 micromoles of glucose-6-phosphate, 680 micromoles of  $\alpha$ -ketoglutaric acid, 1.3 micromoles of TPN, 2.0 micromoles of  $\text{MnCl}_2$ , glycine buffer, pH 7.5 (final concentration 0.018 M), and 6.0 cc. of 0.1 M  $\text{NaHCO}_3$  previously saturated with 100 per cent  $\text{CO}_2$  at 20°. pH of reaction mixture 7.2. After incubation for 1 hour at 20°, protein was precipitated with trichloroacetic acid, the supernatant was treated with barium acetate, and the barium salts were fractionated as described in the text. The fraction of barium salts insoluble in 35 per cent ethanol at pH 7.3 was used. It was dissolved in dilute HCl, the barium was removed with  $\text{Na}_2\text{SO}_4$ , and the solution neutralized to pH 7.3 with dilute NaOH. Aliquots of this solution were incubated with the aconitase preparation described in the section "Methods."

	Time of incubation with aconitase at 25°	d-Isocitric acid found*	Citric acid found†
	min.	$\gamma$	$\gamma$
Experiment 1. 1 cc. aliquot + 1 cc. aconitase	0	182.0	
	5	12.6	
Experiment 2. 3 cc. aliquot + 3 cc. aconitase	0	546.0	45.0‡
	10		450.0

\* Determined enzymatically with isocitric dehydrogenase.

† Determined chemically.

‡ Approximate value; too low for accurate determination.

The equilibrium constant ( $K_e$ ) at pH 7.2 of the carboxylase reaction, *i.e.* Reaction 4, can be calculated from the values of  $K_0$  and  $K_i$ .

$$K_e = \frac{(\text{Oxalosuccinate})}{(\alpha\text{-Ketoglutarate}) (\text{CO}_2)} = \frac{K_0}{K_i} = \frac{1.3 \times 10^{-4}}{0.3} = 0.4 \times 10^{-3}$$

This value is close to that of  $0.2 \times 10^{-3}$  calculated from free energy data by Evans, Vennesland, and Slotin (17) for the reaction catalyzed by oxalacetic carboxylase; *i.e.*,  $\text{oxalacetate} \rightleftharpoons \text{pyruvate} + \text{CO}_2$ .

*Dismutation between Glucose-6-phosphate and  $\alpha$ -Ketoglutarate + CO<sub>2</sub>*—As mentioned above, the synthesis of isocitric acid by reversal of Reaction 5 can be markedly increased by dismutation with glucose-6-phosphate (Reaction 8). Under these conditions enough isocitric acid is obtained for identification. Table II presents such an experiment. After incubation,

the reaction mixture was deproteinized with an equal volume of 10 per cent trichloroacetic acid, and the supernatant was cooled in ice and neutralized with NaOH to pH 6.0. After 5 cc. of 25 per cent barium acetate were added, and sufficient dilute NaOH to bring the pH to 7.3, a small amount of precipitate was centrifuged off and discarded. The supernatant yielded, on addition of 0.5 volume of ethanol, 69.8 mg. of a crude barium salt which contained 2.2 mg. of *d*-isocitric acid as determined enzymatically. Addition of another 0.5 volume of ethanol yielded 69.3 mg. of barium salt, with only traces of isocitric acid. As is shown in Table II, an aliquot of the first barium salt containing 182  $\gamma$  of *d*-isocitric acid, determined en-

TABLE III

*Enzymatic Synthesis of Tricarboxylic Acid by Dismutation between  $\alpha$ -Ketoglutarate + CO<sub>2</sub> and Glucose-6-phosphate, with and without Aconitase (Enzymatic Determination of Isocitric and Total Tricarboxylic Acids)*

0.2 cc. of heart extract and 0.6 cc. of glucose phosphate dehydrogenase (5 mg. per cc.) made up to 2.0 cc. with additions including 25 micromoles of glucose-6-phosphate, 0.15 micromole of TPN, 34 micromoles (5 mg.) of  $\alpha$ -ketoglutaric acid, and 80 micromoles of NaHCO<sub>3</sub>. Gas, 80 per cent N<sub>2</sub> and 20 per cent CO<sub>2</sub>; pH 7.0; temperature 25°. The reaction was started by tipping in  $\alpha$ -ketoglutarate from the side bulb of Warburg vessels after temperature equilibration.

Experiment No.	Sample	Tricarboxylic acid determined	Tricarboxylic acid formed			
			10 min.	20 min.	60 min.	120 min.
1	No aconitase; 2 micromoles MnCl <sub>2</sub>	Isocitric	151	202	270	325
		Total	164	225	320	404
2	" " no MnCl <sub>2</sub>	Isocitric			203	
	" " 2 micromoles MnCl <sub>2</sub>	"			335	
3	0.2 cc. aconitase; 2 micromoles MnCl <sub>2</sub>	"	18	12	30	32
		Total	102	180	408	588

zymatically, lost 169.4  $\gamma$  (or 93 per cent) on incubation with aconitase for 5 minutes; 12.6  $\gamma$  (or 7 per cent) remained unchanged. This is in good agreement with the values obtained by Martius and Leonhardt (7) for the aconitase equilibrium; *i.e.*, 7.7 per cent *d*-isocitric acid and 92.3 per cent citric and *cis*-aconitic acids. A larger aliquot, containing 546  $\gamma$  of *d*-isocitric acid by enzymatic estimation, yielded 450  $\gamma$  of citric acid on incubation with aconitase for 10 minutes; *i.e.*, 82 per cent of the isocitric acid was converted to citric acid. Martius and Leonhardt (7) give 89.2 per cent citric acid at equilibrium.

Various other lines of evidence support the occurrence of Reactions 8 and 9. Table III shows the results of experiments in which the course of

the dismutation, both in the absence and presence of aconitase, was followed by separate enzymatic determination, at various time intervals, of both *d*-isocitric acid and total tricarboxylic acids. Although, as previously mentioned, the heart extracts are largely devoid of aconitase, when relatively large amounts of the extract are used and the incubation periods are long there is some formation of tricarboxylic acids other than isocitric, as may be observed in Experiment 1 of Table III. Experiment 2 shows some increase in the synthesis of isocitric acid when  $MnCl_2$  is added. The fact that substantial amounts of tricarboxylic acid are found in the absence of added  $Mn^{++}$  must be attributed, as discussed above, to the presence of the metal in the crude preparation of *Zwischenferment*. Experiment 3 shows, as was expected from the predicted equilibrium shift, that, in the presence of aconitase, the reaction proceeds faster and farther than in its absence; *i.e.*, more tricarboxylic acid is formed in a given time (*cf.* Experiments 1 and 3, especially at 60 and 120 minutes). The ratio of isocitric acid to total tricarboxylic acid in Experiment 3, from 20 to 120 minutes, is close to that expected for the aconitase equilibrium.

The course of the dismutation can also be readily followed manometrically at pH 7.0 in a bicarbonate-containing medium, owing to the fact that, while the absorption of 1  $CO_2$  equivalent is compensated by liberation from the bicarbonate of another  $CO_2$  equivalent by the third carboxyl group of the tricarboxylic acid formed ( $pK_s$  of citric acid at  $25^\circ$ , 5.4), the oxidation of glucose phosphate to phosphogluconic acid creates an extra carboxyl group which displaces a  $CO_2$  equivalent. Fig. 7 illustrates the course of such a reaction and demonstrates the accelerating action of aconitase. The reaction was started by tipping in  $\alpha$ -ketoglutarate from the side bulbs of the Warburg vessels after temperature equilibration. The keto acid was carefully neutralized to the final pH of the reaction mixture in the main space. In the absence of  $\alpha$ -ketoglutarate there is no reaction, since the oxidation of glucose phosphate ceases as soon as the small catalytic amount of TPN is reduced.

Table IV shows experiments in which the course of the hexose monophosphate- $\alpha$ -ketoglutarate- $CO_2$  dismutation in the presence of aconitase (Reaction 9) was followed for periods up to 6 hours; both the disappearance of  $\alpha$ -ketoglutarate and glucose-6-phosphate and the production of citrate and phosphogluconate were determined at various time intervals. As explained above, the  $CO_2$  evolution, which was followed on an aliquot of the main reaction mixture, is a measure of the formation of phosphogluconate from hexose monophosphate. Glucose phosphate,  $\alpha$ -ketoglutarate, and citrate were determined by chemical methods on aliquots withdrawn from the main reaction mixture at the intervals specified in Table IV. The reaction mixtures were incubated in 100 cc. Erlenmeyer flasks and were equili-



brated with a gas mixture of 80 per cent nitrogen and 20 per cent  $\text{CO}_2$  throughout the incubation period. It will be observed that while there is a fairly good agreement between the  $\text{CO}_2$  evolution (formation of phosphogluconic acid), on the one hand, and the removal of  $\alpha$ -ketoglutarate and glucose-6-phosphate, on the other, the amounts of citric acid found fall short of the expected values, even if they are increased by 10 or 11 per cent

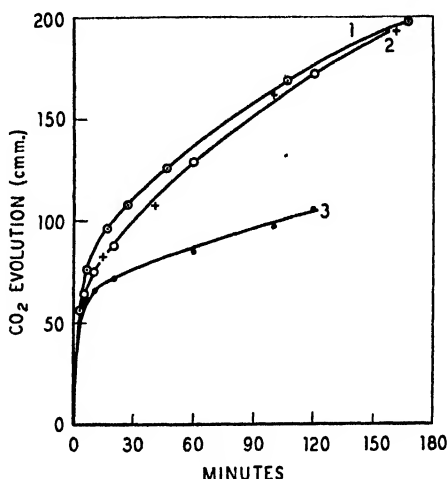


FIG. 7. Time course of the dismutation between  $\alpha$ -ketoglutarate +  $\text{CO}_2$  and glucose-6-phosphate, with and without aconitase, as determined by the liberation of carbon dioxide from bicarbonate caused by formation of phosphogluconic acid. For each experiment 0.1 to 0.2 cc. of heart extract and 0.6 cc. of glucose phosphate dehydrogenase (5 mg. per cc.) were made up to a volume of 2.0 cc. with additions including 0.15 micromole of TPN, 2.0 micromoles of  $\text{MnCl}_2$ , and 80 micromoles of  $\text{NaHCO}_3$ . Curve 1, with 0.2 cc. of aconitase, 64 micromoles of glucose-6-phosphate, and 68 micromoles of  $\alpha$ -ketoglutarate. Curve 2, with 0.2 cc. of aconitase, 25 micromoles of glucose-6-phosphate, and either 34 (O) or 27.5 (X) micromoles of  $\alpha$ -ketoglutarate. Curve 3, with 25 micromoles of glucose-6-phosphate and 34 micromoles of  $\alpha$ -ketoglutarate but *without aconitase*. Gas, 80 per cent  $\text{N}_2$  and 20 per cent  $\text{CO}_2$ ; pH 7.0. Temperature,  $25^\circ$ . The reaction was started by tipping in  $\alpha$ -ketoglutarate from the side bulb of Warburg vessels after temperature equilibration.

to obtain the value for total tricarboxylic acid. Owing to the crude condition of the enzymes used, some side reactions must occur which are responsible for the observed discrepancy. The nature of these reactions has not yet been investigated.

#### DISCUSSION

The experimental results presented in this paper indicate the possibility of extensive biosynthesis of tricarboxylic acids through fixation of carbon

dioxide by  $\alpha$ -ketoglutaric acid. The occurrence of such a fixation is due to the reversibility of the reaction  $\text{oxalosuccinate} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{CO}_2$ . While the equilibrium of this reaction is far to the right, *i.e.* in the direction of decarboxylation, it can be shifted in the opposite direction through reduction of oxalosuccinate to *d*-isocitrate by reduced triphosphopyridine nucleotide (TPN<sub>red.</sub>) in the presence of isocitric dehydrogenase. If the oxidized TPN (TPN<sub>ox.</sub>) thereby formed is reduced by an independent dehydrogenase system of suitable oxidation-reduction potential, there occurs

TABLE IV

*Dismutation between  $\alpha$ -Ketoglutarate + CO<sub>2</sub> and Glucose-6-phosphate in Presence of Aconitase (Manometric and Chemical Estimations)*

1.0 cc. of heart extract, 6.0 cc. of glucose phosphate dehydrogenase (5 mg. per cc.), and 2.0 to 4.0 cc. of aconitase made up to 20 cc. with additions including 1.3 micromoles of TPN, 20 micromoles of MnCl<sub>2</sub>, and 800 micromoles of NaHCO<sub>3</sub>. Gas, 80 per cent N<sub>2</sub> and 20 per cent CO<sub>2</sub>; pH 7.0; temperature, 25°. Evolution of CO<sub>2</sub> determined manometrically with 2.0 cc. aliquots of reaction mixtures. All values expressed in micromoles.

Experiment No.	Glucose phosphate added	Ketoglutarate added	Incubation time	CO <sub>2</sub> evolved	Citric acid formed	Ketoglutaric acid removed	Glucose phosphate removed
			hrs.				
1	640	680	2	81.2	53.6	84.4	
			6		101.6	140.0	156.0
2	250	275	0.5	27.2	11.2	28.7	
			1	36.5	20.8		39.0
			2	46.0	30.5	46.7	
			3	59.0	38.2	57.0	
			6		44.0	106.0	102.0
3	250	275	0.5	46.0	26.3	51.2	
			1	51.0	35.0	59.0	
			2	77.5	52.8	87.2	
			3	90.0	60.0	114.0	104.0
			4.5		62.5	127.0	125.0

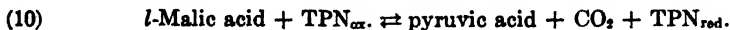
a further shifting of equilibrium in favor of CO<sub>2</sub> fixation. The successful use of the TPN-specific glucose-6-phosphate dehydrogenase system for such a purpose has already been discussed in the foregoing section. The oxidation-reduction potential of this system (reductant) is given by Barron (18) as  $E'_0 = -0.43$  volt at pH 7, while that of the isocitric  $\rightleftharpoons$  oxalosuccinic system (the oxidant) has been estimated, from the approximate value of the equilibrium constant reported in this paper, to lie in the neighborhood of  $-0.30$  volt (19). Essentially, the exergonic oxidation of glucose phosphate is made use of here to drive the endergonic carboxylation of  $\alpha$ -ketoglutaric acid. An approximate estimate of the free energy change of this

carboxylation, from its equilibrium constant, gives a value of about +4000 calories (19). Further disturbance of the equilibrium with shifting in the direction of CO<sub>2</sub> fixation occurs, as shown in the previous section, through isomerization of *d*-isocitric to citric acid in the presence of aconitase, owing to the fact that the equilibrium of such a reaction is markedly in favor of citric acid. The free energy changes of the partial reactions involved in this system of CO<sub>2</sub> fixation have been discussed elsewhere (19).

The type of carboxylation reaction here considered ( $\beta$ -carboxylation) is to be distinguished from "reductive" carboxylation which, as Lipmann (20) discovered, occurs by reversal of the oxidative decarboxylation of  $\alpha$ -keto acids. The latter reaction involves a much greater change of free energy which is supplied in part by energy-rich phosphate through the generation of an acyl phosphate bond by reaction with ATP (20). In this connection it should be emphasized that the reactions discussed in this paper, whether partial or over-all, proceed in either direction in the absence of inorganic phosphate and ATP. Further, no generation of phosphate bonds is connected with the oxidation of isocitric acid to  $\alpha$ -ketoglutaric acid and CO<sub>2</sub> (14).

It is tempting to speculate on the possibility that the tricarboxylic acid formed by CO<sub>2</sub> fixation might break down, possibly by reversal of its as yet poorly understood synthesis from dicarboxylic acid and a C<sub>2</sub> derivative. If so, the tricarboxylic acid cycle of Krebs as a whole might be reversible. As discussed elsewhere (19), such mechanisms might conceivably be at work in photosynthesis and chemosynthesis.

A question of great interest is whether isocitric dehydrogenase and oxalosuccinic carboxylase are distinct enzymes and can catalyze the over-all Reaction 5 acting together, or whether this reaction, *i.e.* the reversible oxidative decarboxylation of *d*-isocitric acid to  $\alpha$ -ketoglutaric acid and CO<sub>2</sub>, is catalyzed by a single enzyme. While it would appear that the evidence presented in this paper favors the former possibility, partial purification of the heart extracts has so far failed to separate the two activities. The need for caution on this point was stressed by the finding that the reversible conversion of *l*-malate to pyruvate and CO<sub>2</sub>, a reaction identical in nature to Reaction 5, is not catalyzed by a combination of malic dehydrogenase (21) and a partially purified oxalacetic carboxylase from *Micrococcus lysodeikticus* in the presence of either DPN or TPN and manganous ions; this observation led to the isolation of an enzyme from pigeon liver which, in the presence of Mn<sup>++</sup>, catalyzes the reversible Reaction 10 (22).



Extensive purification of the pigeon liver enzyme<sup>2</sup> has shown that it also catalyzes the decarboxylation of oxalacetate to pyruvate and CO<sub>2</sub> (but not

<sup>2</sup> Ochoa, S., Mehler, A. H., and Kornberg, A., unpublished.

the reduction of oxalacetate to *l*-malate by  $\text{TPN}_{\text{red}}$ ), and strongly suggests that a single enzyme is involved in the catalysis of Reaction 10. Thus, in the case of dicarboxylic acids, we have a malic dehydrogenase, an oxalacetic carboxylase, and, in all probability, another single enzyme that catalyzes Reaction 10. Under these conditions, the possibility that such a situation may also obtain in the case of tricarboxylic acids cannot be discarded until a decision can be made through purification of the enzymes involved; such work is now in progress.

### Methods

#### Enzyme Preparations

*Isocitric Dehydrogenase*—The preparation of pig heart extracts containing isocitric dehydrogenase and oxalosuccinic carboxylase has been described in a previous paper (12). Partial purification of the isocitric dehydrogenase was obtained as follows: The undialyzed phosphate extract was cooled to  $0^\circ$  and treated with sufficient calcium acetate solution to precipitate the orthophosphate. The calcium phosphate was centrifuged off at  $0^\circ$  and the supernatant (pH 5.6) was brought to pH 6.2 with  $\text{Na}_2\text{CO}_3$  and to 60 per cent saturation at  $0^\circ$  with solid ammonium sulfate; the precipitate was filtered off and discarded. The enzyme precipitates as the ammonium sulfate is increased to 65 per cent of saturation. The precipitate was dissolved in water and refractionated with ammonium sulfate. The fraction between 40 and 50 per cent ammonium sulfate saturation was dissolved in water, brought to pH 8.0 with dilute ammonium hydroxide, and treated with a suspension of alumina gel C-7; the alumina was centrifuged off and discarded. This procedure led to a 4-fold increase of the specific activity of isocitric dehydrogenase as compared with that of the initial extract. The activity determinations were based on the over-all Reaction 5; the rate of reduction of TPN, in the presence of the enzyme,  $\text{Mn}^{++}$ , and an excess of isocitric acid, was determined spectrophotometrically. Under these conditions, the reduction of TPN follows a first order reaction. Since the initial rate of reduction of TPN was found, within certain limits, to be proportional to the concentration of enzyme, an enzyme unit was defined as the amount of enzyme causing an increase in optical density (at  $340\text{ m}\mu$ ) of 0.01 in the 1st minute. The course of a fractionation is summarized in Table V.

The purified preparations also catalyzed the decarboxylation of oxalosuccinic acid.

*Aconitase*—The activity of this enzyme was also determined spectrophotometrically by measuring the rate of reduction of TPN, in the presence of an excess of isocitric dehydrogenase (containing oxalosuccinic carboxylase) and  $\text{Mn}^{++}$ , with either citric acid or *cis*-aconitic acid as substrate.

In the absence of aconitase there is no reaction. In the presence of aconitase, and under certain conditions which have been worked out by Dr. A. Kornberg in this laboratory,<sup>3</sup> the reaction is zero order over most of its course.

A crude but very active aconitase preparation was obtained as follows: The ventricular muscle of five pig hearts was washed with water, cooled in ice, and minced cold. The mince (about 880 gm.) was extracted for 1 hour, at 3–4°, with 1 liter of distilled water with occasional stirring; the mixture was pressed through gauze and the fluid centrifuged at 0°. The following operations were carried out at 0°. To the extract (810 cc.) were added 372 gm. of solid ammonium sulfate (65 per cent saturation) under vigorous stirring and, after standing overnight, the precipitate was filtered off and dissolved in 100 cc. of distilled water. A small amount of insoluble material was centrifuged off. The solution (134 cc.) was dialyzed for about 70

TABLE V  
*Partial Purification of Isocitric Dehydrogenase*

Step	Specific activity	Yield
	<i>units per mg. protein</i>	
Extract.....	167	100
1st (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation (0.6–0.65 saturation).....	436	14
2nd “ “ (0.4–0.5 saturation).....	500	6.5
Supernatant from Al(OH) <sub>3</sub> .....	670	4.2

hours against 2 liters of 55 per cent saturated ammonium sulfate; the precipitate was centrifuged off at 15,000 R.P.M. and discarded. To the solution (56 cc.) were added 8.6 cc. of saturated ammonium sulfate (degree of saturation with ammonium sulfate, 65 per cent). The precipitate was centrifuged off at high speed and dissolved in 30 cc. of water, and the solution was dialyzed for 3 hours against running tap water at 10° and centrifuged at high speed to remove a small amount of precipitate formed on dialysis. The specific activity of aconitase in this solution is only about twice as high as in the initial extract, the main advantage achieved being a much higher concentration of the enzyme. The enzyme is very unstable and loses activity rather rapidly even when stored at 0°.

#### *Chemical Preparations*

*Triphosphopyridine Nucleotide*—TPN was prepared essentially according to the method of Warburg, Christian, and Griesse (23). The purity of

<sup>3</sup> Kornberg, A., unpublished.

the preparation was determined spectrophotometrically with isocitric dehydrogenase in the test system of Reaction 5. This is possible because the equilibrium of such a reaction is so far to the right that, with an excess of isocitric acid, the nucleotide present is practically reduced to completion. With the value for the molecular absorption coefficient of  $\text{TPN}_{\text{red}}$ , given by Warburg (10),  $\beta = \ln(I_0/I)/c \times d = 1.3 \times 10^7$  (sq. cm. per mole), the purity of the desiccator-dried preparation was 54.6 per cent. This was in fairly good agreement with the value of 50.4 per cent calculated from its phosphorus content, assuming that the molecular weight of TPN is 743. The preparation was free from inorganic phosphate, but its ribose content, determined by the method of Mejbaum (24), was higher than theory (ribose to P ratio, found, 1.05; theory, 0.67). The preparation was free from DPN as tested with crystalline triosephosphate dehydrogenase (25), in the optical test system of Warburg and Christian, in the presence of arsenate (26).<sup>4</sup> Its almost complete freedom from adenosine mono- and polyphosphates was established by a sensitive spectrophotometric test.<sup>3</sup>

*Isocitric Acid*—*dl*-Isocitric acid was prepared by the method of Fittig and Miller (27). The crude material obtained after decomposition of the barium salt was washed with a little cold ethyl acetate, dissolved in just the required amount of ethyl acetate at room temperature, and the solution was treated with petroleum ether to a permanent turbidity; the isocitric acid crystallized on standing in the ice box. The material melted at 125° with softening between 90–100°; 69.8 mg. required 10.5 cc. of 0.1 N NaOH for neutralization (in ice water) and the same amount after being boiled, indicating freedom from isocitric acid lactone. One-half of the synthetic isocitric acid reacts in the isocitric dehydrogenase test; this indicates that the product contains only the naturally occurring acid and its enantiomorph and is, therefore, *dl*-isocitric acid (28). By titration with TPN, in the optical test of Reaction 5, the purity of the preparation was estimated to be 99 per cent.

*Other Preparations*—Glucose-6-phosphate was prepared synthetically by the method of Levene and Raymond (29). After being dried *in vacuo* over  $\text{P}_2\text{O}_5$ , the barium salt contained 7.6 per cent P (calculated for  $\text{C}_6\text{H}_{11}\text{O}_6\text{PBa}$ , 7.84 per cent). *cis*-Aconitic anhydride was prepared by the method of Malachowski and Maslowski (30). The anhydride (m.p. 75°) was dissolved in warm ethyl acetate (previously saturated with water at room temperature) and the acid crystallized by addition of chloroform to a permanent turbidity, as described by Krebs and Eggleston (31); it melted at 125°. Oxalosuccinic and  $\alpha$ -ketoglutaric acids were prepared as previously described (11, 32).

<sup>4</sup> I am indebted to Dr. E. Racker for this determination.

### Analytical Methods

**Enzymatic**—In the presence of an excess of TPN, *d*-isocitric acid can be determined by the optical test system of Reaction 5. Since, as previously discussed, the equilibrium of this reaction lies very far to the right, for all practical purposes all of the *d*-isocitric acid present will react, 1 mole of TPN being reduced by 1 mole of *d*-isocitric acid. In the absence of significant amounts of  $\text{HCO}_3^-$  ions,  $\alpha$ -ketoglutaric acid does not appreciably interfere with the estimation, even if present in a relatively large molar excess (20- to 30-fold) with relation to isocitric acid. The reaction mixtures whose *d*-isocitric acid content is to be determined are treated with 0.1 volume of 0.2 M acetate buffer, pH 4.0, heated for 2 minutes in a boiling water bath, and cooled; the coagulated protein is removed by centrifugation. They can also be deproteinized with trichloroacetic acid, filtered, and neutralized. In either case the supernatants, which should be clear, are practically free from  $\text{CO}_2$ . Suitable aliquots, containing a few micrograms of isocitric acid, are used for the estimation. The determination is carried out in the presence of  $\text{Mn}^{++}$ , as described above, with sufficient heart enzyme to complete the reaction in a few minutes. The final volume of reaction mixture in the absorption cells was 3.0 cc.,  $d = 1.0$  cm. After a zero time reading of the optical density at 340  $m\mu$  is taken, the reaction is usually started by adding  $\text{TPN}_{\text{ox}}$ ; readings are then taken at frequent intervals until there is no further increase in optical density. Our preparations of  $\text{TPN}_{\text{ox}}$  show a very small absorption at 340  $m\mu$ , which is subtracted from the final reading. Since the molecular absorption coefficient of  $\text{TPN}_{\text{red}}$  is  $\beta = \ln(I_0/I)/c \times d = 1.3 \times 10^7$  (sq. cm. per mole) (10), or the extinction coefficient  $\alpha = \log(I_0/I)/c \times d = 0.5644 \times 10^7$  (sq. cm. per mole), for  $\log(I_0/I) = 0.01$ , the concentration  $c$  of  $\text{TPN}_{\text{red}}$ , or *d*-isocitric acid will be  $c = (0.01/0.5644) \times 10^{-7}$ , or  $0.01772 \times 10^{-7}$  mole per cc. and, for a volume of 3.0 cc.,  $0.0532 \times 10^{-7}$  mole or (since the molecular weight of isocitric acid is 192) 1.02  $\gamma$  of isocitric acid. The quantity in micrograms of isocitric acid in the aliquot used, under our experimental conditions, is thus obtained by multiplying by 102 the maximum  $\Delta \log(I_0/I)$  reached. This method is rapid, sensitive, and quite specific for *d*-isocitric acid, even if the enzyme preparation used is the crude dialyzed heart extract. Except for the presence of a very weak glutamic dehydrogenase, these extracts are free of dehydrogenases reacting with TPN other than isocitric dehydrogenase (*i.e.*, glucose and glucose phosphate dehydrogenases and the recently described enzyme catalyzing the reversible oxidative decarboxylation of malic acid (22)). Since the equilibrium of the glutamate  $\rightleftharpoons$   $\alpha$ -ketoglutarate +  $\text{NH}_3$  system is far to the side of glutamate (33), glutamic acid does not interfere unless present in large amounts.

Because the heart extracts are practically devoid of aconitase, tricarbox-

ylic acids other than *d*-isocitric acid are not determined by the above method. However, *cis*-aconitic and citric acids can be roughly determined by addition of aconitase to the reaction mixture once the reaction with isocitric acid (if any is present) is complete. Under these conditions, *cis*-aconitic acid reacts very rapidly and reduces 1 TPN equivalent. On the other hand, the reaction with citric acid is very slow, owing to the unfavorable equilibrium, and, even after 30 minutes, only about 80 per cent of the citric acid has reacted. Thus, for approximate estimations of total tricarboxylic acids, after determining *d*-isocitric acid, 0.05 to 0.1 cc. of aconitase is added and a few readings are taken. If the optical density increases further, indicating the presence of other tricarboxylic acids, the reaction mixture is set aside and its optical density at 340 m $\mu$  is determined again after 30 or more minutes until there is no further significant change.

*Chemical*—Citric acid was determined in trichloroacetic acid filtrates by the method of Goldberg and Bernheim (34). Appropriate blanks and at least two controls with known amounts of citric acid were always run simultaneously with each series of determinations. Glucose-6-phosphate was determined in tungstic acid filtrates by its reducing power, since it was the only reducing substance present in the reaction mixtures in which progress of Reaction 9 was followed. The method of Shaffer and Somogyi (35) was used and the copper reagent was standardized with known amounts of glucose-6-phosphate.  $\alpha$ -Ketoglutaric acid was determined in trichloroacetic acid filtrates by the method of Friedemann and Haugen (36).

#### SUMMARY

Crude solutions of isocitric dehydrogenase from pig heart catalyze, in the presence of TPN and manganous ions, the reversible conversion of *d*-isocitric acid to  $\alpha$ -ketoglutaric acid and carbon dioxide. The reaction involves two reversible steps: (a) *d*-isocitric acid + TPN<sub>ox.</sub>  $\rightleftharpoons$  oxalosuccinic acid + TPN<sub>red.</sub>, and (b) oxalosuccinic acid  $\rightleftharpoons$   $\alpha$ -ketoglutaric acid + CO<sub>2</sub>. Step (b) requires Mn<sup>++</sup> but step (a) does not. The available evidence suggests that steps (a) and (b) are catalyzed by two distinct enzymes, isocitric dehydrogenase and oxalosuccinic carboxylase respectively, but final decision on this point must await purification of the enzymes involved.

The equilibrium constant of the over-all reaction



$$\left( K = \frac{(d\text{-Isocitrate}) (\text{TPN}_{\text{ox.}})}{(\alpha\text{-Ketoglutarate}) (\text{H}_2\text{CO}_2) (\text{TPN}_{\text{red.}})} \right)$$

is about  $1.3 \times 10^{-4}$ , indicating that equilibrium lies very far in the direction of decarboxylation.

The equilibrium can be markedly shifted in the direction of CO<sub>2</sub> fixation



by combination with the glucose-6-phosphate dehydrogenase system. In this case, TPN is reduced according to the reaction, glucose-6-phosphate +  $\text{TPN}_{\text{ox.}} \rightarrow$  6-phosphogluconate +  $\text{TPN}_{\text{red.}}$ . The net result is the dismutation, glucose-6-phosphate +  $\alpha$ -ketoglutarate +  $\text{CO}_2 =$  6-phosphogluconate + *d*-isocitrate. The isocitrate formed was isolated as a crude barium salt and identified enzymatically with aconitase.

When the dismutation is carried out in the presence of aconitase, further shifting of equilibrium occurs in the direction of  $\text{CO}_2$  fixation, owing to the fact that the equilibrium between isocitric, *cis*-aconitic, and citric acids, whose establishment is catalyzed by aconitase, is predominantly in favor of citric acid.

The above results suggest that the enzyme system oxalosuccinic carboxylase-isocitric dehydrogenase-aconitase may play an important part in the biological utilization of carbon dioxide.

I am indebted to Dr. Erwin Haas for a generous gift of yeast glucose-6-phosphate dehydrogenase and to Dr. Milton Levy for many helpful suggestions. My thanks are also due to Dr. Erna Weisz-Tabori for much help with chemical preparations and estimations, and to Mr. Morton C. Schneider for help with the enzyme preparations.

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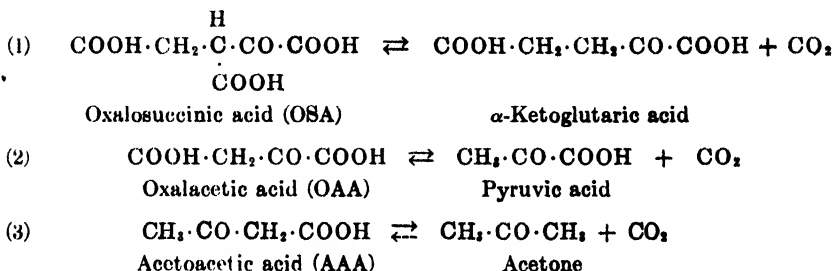
# SPECTROPHOTOMETRIC STUDIES ON THE DECARBOXYLATION OF $\beta$ -KETO ACIDS\*

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The decarboxylation of some  $\beta$ -keto acids of biological importance in oxidative metabolism is catalyzed by specific enzymes. Some of these reactions are reversible and, in this way, play an important part in the biological assimilation of carbon dioxide. Reactions 1, 2, and 3 are catalyzed by specific enzymes from various sources.



Reaction 1 is catalyzed by an enzyme widely distributed in animal tissues and present in some plants (1). Oxalacetic carboxylase, which catalyzes Reaction 2, has been found in bacteria (2), pigeon liver (3), and, to some extent, in other animal tissues as well as in plants (4). Mehler *et al.*<sup>1</sup> have partially purified this enzyme from *Micrococcus lysodeikticus*. Acetoacetic carboxylase (Reaction 3) has been obtained from acetone bacteria in highly purified form (5).

It is well known that the above  $\beta$ -keto acids are more or less unstable and undergo spontaneous decarboxylation in aqueous solution. Their stability decreases in the following order: acetoacetic, oxalacetic, oxalosuccinic.

Krebs (6) observed that the decarboxylation of OAA, but not that of AAA, is catalyzed by polyvalent cations, notably by  $\text{Zn}^{++}$ ,  $\text{Cu}^{++}$ ,  $\text{Fe}^{++}$ ,

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<sup>1</sup> Mehler, A. H., Kornberg, A., Grisolia, S., and Ochoa, unpublished.

$\text{Fe}^{+++}$ , and  $\text{Al}^{+++}$ .  $\text{Ca}^{++}$ ,  $\text{Ba}^{++}$ ,  $\text{Mg}^{++}$ , and  $\text{Mn}^{++}$  were much less effective. OSA was found in this laboratory to react toward cations in the same way as OAA (1). Speck<sup>2</sup> has recently added  $\text{Cd}^{++}$ ,  $\text{Co}^{++}$ , and  $\text{Ni}^{++}$  to the list of cations that accelerate the decarboxylation of OAA.

Although  $\text{Mn}^{++}$ , when present alone, is a poor catalyst of the decarboxylation of OSA and OAA, it is required by the specific carboxylases for activity (1-4).  $\text{Mg}^{++}$  is much less effective. Acetoacetic carboxylase does not seem to require a metal for activity (5).

The interaction of metals,  $\beta$ -keto acids, and their carboxylases had thus far been studied by manometric methods. By the use of spectrophotometric methods we have been able to gain some understanding of the underlying mechanisms of these reactions. Our results indicate that cations like  $\text{Al}^{+++}$  form complexes with OSA, OAA, and AAA in the absence of a protein. The complexes formed by  $\text{Al}^{+++}$  with OSA, and OAA are unstable and decarboxylate at a rate faster than that of spontaneous decarboxylation of the  $\beta$ -keto acids, but the AAA complex is stable. While  $\text{Mg}^{++}$  and  $\text{Mn}^{++}$  also form complexes with OSA and OAA in the absence of protein, the extent of this complex formation or the lability of the complex is too slight to effect rapid decarboxylation. Each of these various complexes has a characteristic absorption spectrum in the ultraviolet. Our data also suggest that the effective catalysis of decarboxylation achieved by oxalosuccinic carboxylase in the presence of  $\text{Mn}^{++}$  is largely the result of an acceleration of the rate of complex formation. In the case of oxalacetic carboxylase, it appears that both the rate of complex formation and breakdown are influenced.

### Results

*Effect of Metals*—The ultraviolet absorption spectra at pH 7.3 of the Na salts of OSA, OAA, AAA, and of  $\alpha$ -ketoglutaric and pyruvic acids (the decarboxylation products of OSA and OAA respectively) are shown in Fig. 1. The absorption of acetone, which is negligible, is not shown. It is evident that within a given wave-length range the decarboxylation of OSA and OAA should result in a decrease of the optical density of the solution. However, the addition of  $\text{Al}^{+++}$  which, as pointed out above, markedly increases the rate of decarboxylation of these acids, results at first not in a decrease, but in a sharp increase of the optical density, followed by a rapid decrease toward the level corresponding to the products of decarboxylation.<sup>3</sup> This is illustrated in Fig. 2.  $\text{Al}^{+++}$  has no effect on the absorption of  $\alpha$ -ketoglutaric and pyruvic acids. In the case of AAA there is also a sharp increase in

<sup>2</sup> Speck, J. F., personal communication.

<sup>3</sup> The apparent failure of the optical density to drop to the levels corresponding to complete decarboxylation is due to turbidity contributed by aluminum hydroxide.

optical density upon the addition of  $\text{Al}^{+++}$ . However, the optical density remains stationary once the maximum is reached. It will be recalled that  $\text{Al}^{+++}$  does not cause a decarboxylation of this acid. When the maximum optical densities obtained at various wave-lengths after addition of  $\text{Al}^{+++}$

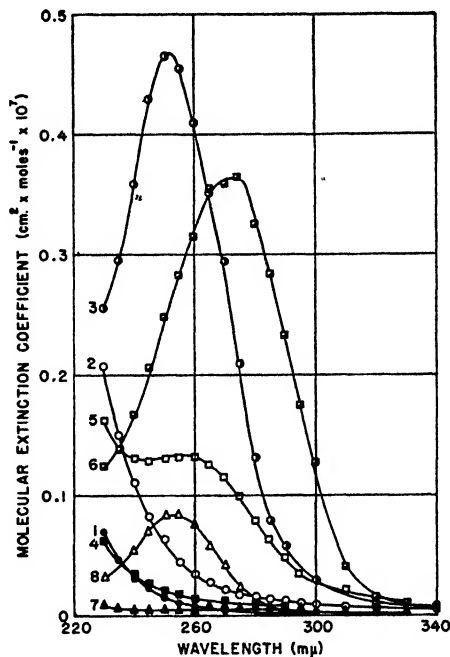


FIG. 1. Ultraviolet absorption spectra of  $\beta$ -keto acids and their aluminum complexes. Solutions of keto acids adjusted with NaOH to pH about 7.3. Final concentrations,  $5 \times 10^{-4}$  M oxalosuccinate and  $\alpha$ -ketoglutarate,  $2.5 \times 10^{-4}$  M oxalacetate, pyruvate, and  $\text{Al}_2(\text{SO}_4)_3$ ,  $2.1 \times 10^{-3}$  M acetoacetate. Total volume, 2.0 cc. Quartz cells;  $d = 0.5$  cm. Curve 1,  $\alpha$ -ketoglutarate either with or without  $\text{Al}^{+++}$ ; Curve 2, oxalosuccinate; Curve 3, oxalosuccinate and  $\text{Al}^{+++}$ ; Curve 4, pyruvate either with or without  $\text{Al}^{+++}$ ; Curve 5, oxalacetate; Curve 6, oxalacetate and  $\text{Al}^{+++}$ ; Curve 7, acetoacetate; Curve 8, acetoacetate and  $\text{Al}^{+++}$ . Curves 3, 6, and 8 were obtained by plotting the maximum densities reached at each wave-length. The expression "molecular extinction coefficient" is used only in terms of keto acid concentration and not in terms of keto acid-metal complex concentration, which is unknown.

to aqueous solutions of each of the three  $\beta$ -keto acids are plotted, Curves 3, 6, and 8 (Fig. 1) are obtained.

These results indicate the  $\text{Al}^{+++}$  forms complexes, having characteristic

This is probably a result of increased pH during decarboxylation. The addition of small amounts of acid at the conclusion of the reaction removed the turbidity and resulted in conformity of the optical densities to the theoretical conversion of the  $\beta$ -keto acids to the corresponding lower  $\alpha$ -keto acids.

ultraviolet absorption spectra, with each of the above  $\beta$ -keto acids. They further indicate that the aluminum complexes of OSA and OAA are unstable and that the keto acid in the complex is readily decarboxylated. On the other hand, the aluminum complex of AAA is stable. It should be recalled that  $\text{Fe}^{+++}$ , which gives a red color with the three  $\beta$ -keto acids, accelerates the decarboxylation of OSA and OAA as effectively as does  $\text{Al}^{+++}$ . This is undoubtedly due to the formation of complexes with absorption in the vis-

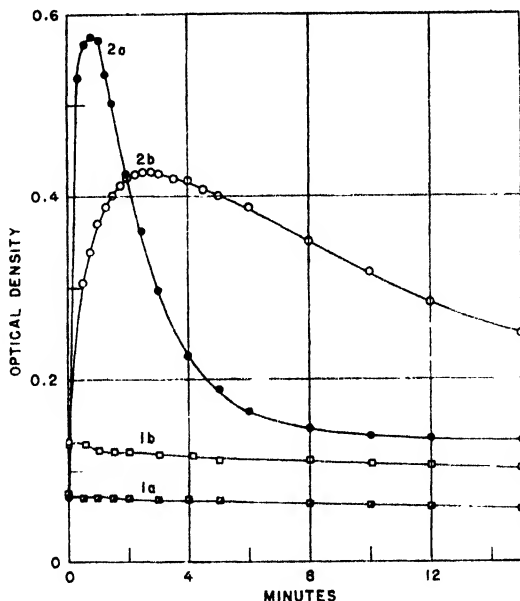


FIG. 2. Time course of spectral changes of oxalosuccinic and oxalacetic acids in the absence and presence of aluminum ions. Final concentrations,  $2.5 \times 10^{-4}$  M keto acid. Wave-length, oxalosuccinate 252  $\mu$ , oxalacetate 270  $\mu$ . Other data as in Fig. 1. Curve 1a, oxalosuccinate; Curve 2a, oxalosuccinate and  $1.25 \times 10^{-4}$  M  $\text{Al}_2(\text{SO}_4)_3$ ; Curve 1b, oxalacetate; Curve 2b, oxalacetate and  $1.25 \times 10^{-4}$  M  $\text{Al}_2(\text{SO}_4)_3$ .

ible range of the spectrum. The fact that the color given by  $\text{Fe}^{+++}$  with OSA and OAA fades rapidly, whereas that given with AAA is stable, demonstrates the similar stability of the  $\text{Fe}^{+++}$  and  $\text{Al}^{+++}$  complexes.

Since  $\text{Mn}^{++}$  is required for the activity of oxalosuccinic and oxalacetic carboxylases, its effect on the ultraviolet absorption of the keto acids was determined. The changes produced, although slight, are definite (Fig. 3; cf. Curves 1 and 2, 4 and 5).  $\text{Mn}^{++}$  does not alter the absorption by  $\alpha$ -keto-glutaric and pyruvic acids. The effect of  $\text{Mg}^{++}$  on the spectrum of OSA and OAA is qualitatively similar to that of equimolar concentrations of  $\text{Mn}^{++}$ , although somewhat less marked. When followed in time at a given

wave-length, the effect of  $Mn^{++}$  on OSA is similar to, but much less pronounced than, that of  $Al^{+++}$ ; there is an increase followed by a decrease of the optical density (Fig. 4, Curve 1). Hence Curves 2 and 5 in Fig. 3 were obtained by plotting against the wave-length the maximum optical density reached after addition of  $Mn^{++}$  to the keto acids, as was done in the case of  $Al^{+++}$ . Therefore, Curves 2 and 5 of Fig. 3 represent the approximate absorption spectra of the manganese complexes of OSA and OAA respectively.

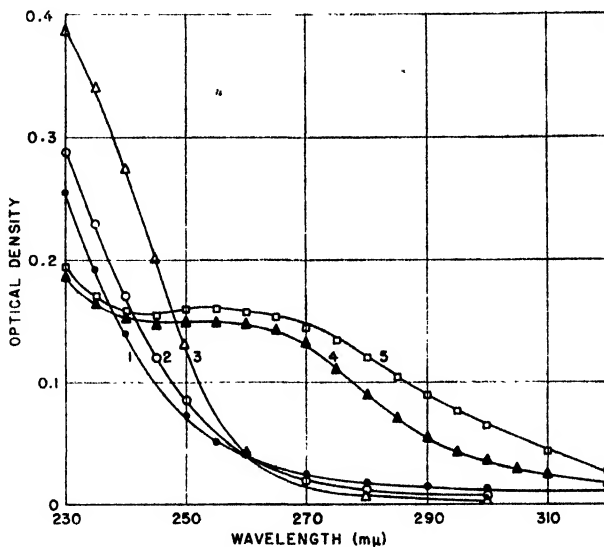


FIG. 3. Ultraviolet absorption spectra of  $\beta$ -keto acids and their manganese complexes. Final concentrations,  $1.67 \times 10^{-4} M$  oxalosuccinate,  $2.5 \times 10^{-4} M$  oxalacetate. Total volume, oxalosuccinate samples 3.0 cc. ( $d = 1.0$  cm.), oxalacetate samples 2.0 cc. ( $d = 0.5$  cm.). Other data as in Fig. 1. Curve 1, oxalosuccinate; Curve 2, oxalosuccinate and  $1.67 \times 10^{-4} M$   $MnCl_2$ ; Curve 3, oxalosuccinate,  $MnCl_2$ , 0.02 cc. of enzyme (pig heart extract), and  $0.134 M$   $KCl$ ; Curve 4, oxalacetate; Curve 5, oxalacetate and  $5 \times 10^{-4} M$   $MnCl_2$ . Curves 2, 3, and 5 were obtained by plotting the maximum densities reached at each wave-length.

*Oxalosuccinic Carboxylase*—As mentioned above, this enzyme has been found in manometric studies to require  $Mn^{++}$  for activity,  $Mg^{++}$  being ineffective at similar concentrations (1). Spectrophotometrically, addition of oxalosuccinic carboxylase to OSA results in a slight increase in optical density at  $240 m\mu$ , followed by a gradual decrease.<sup>4</sup> With both enzyme and

<sup>4</sup> This effect of the enzyme, in the absence of added  $Mn^{++}$ , may be due to the presence of small amounts of this metal in the crude enzyme preparations used. The effect largely disappears after the enzyme is dialyzed against pyrophosphate at pH 8.4, followed by dialysis against  $KCl$  to remove the pyrophosphate.



$Mn^{++}$  present, there is a large and rapid increase in density, succeeded by a prompt decline to a value approximating that of  $\alpha$ -ketoglutaric acid. These results are shown in Fig. 4 (Curves 1, 2, and 3). Potassium chloride (0.134 M) inhibits the increase in density associated with the addition of

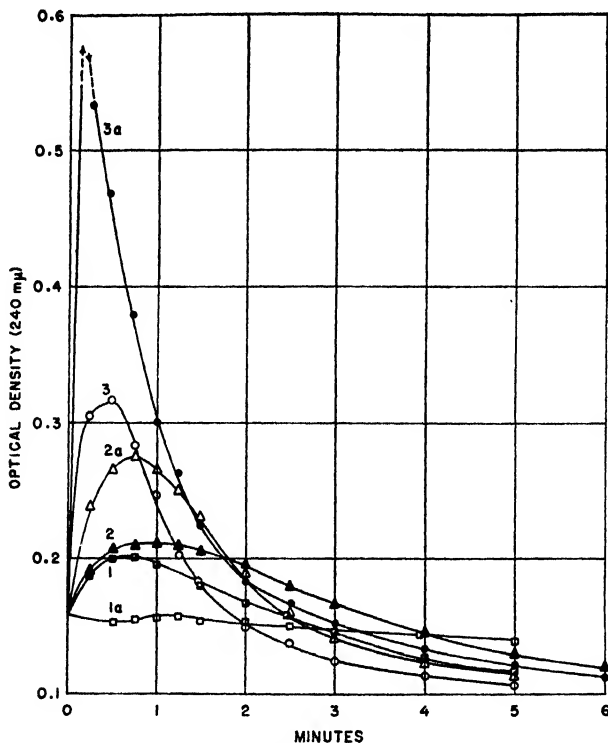


FIG. 4. Spectral changes connected with the enzymatic decarboxylation of oxalosuccinic acid. Final concentrations,  $1.67 \times 10^{-4}$  M oxalosuccinate,  $1.67 \times 10^{-4}$  M  $MnCl_2$ . Total volume of reaction mixtures, 3.0 cc. ( $d = 1.0$  cm.). Wave-length, 240 mμ. Other data as in Fig. 1. Curve 1, oxalosuccinate and  $MnCl_2$ ; Curve 2, oxalosuccinate and 0.1 cc. of enzyme (pig heart extract); Curve 3, oxalosuccinate,  $MnCl_2$ , and enzyme. Curves 1a, 2a, and 3a, the same as Curves 1, 2, and 3, respectively, but each with 0.134 M KCl in addition.

$Mn^{++}$  alone (cf. Curves 1 and 1a, Fig. 4), but it greatly augments the changes resulting from the addition of oxalosuccinic carboxylase with  $Mn^{++}$  and, to a lesser extent, those caused by the carboxylase without  $Mn^{++}$ . These striking effects of potassium chloride are shown in Fig. 4 (cf. Curves 2 and 2a, 3 and 3a); the actual peak of Curve 3a was reached too early to permit accurate measurement. The action of potassium chloride is probably an unspecific effect of increased ionic strength, since the effects of potassium

bromide (0.134 M) and sodium chloride (0.134 M) were indistinguishable from those of potassium chloride. The manometric data in Table I verify the stimulatory effect of potassium chloride on oxalosuccinic carboxylase activity.

The ultraviolet spectrum obtained by plotting maximum optical density against wave-length after the addition of OSA to carboxylase and  $Mn^{++}$  is shown in Fig. 3 (Curve 3). This curve is closely related to that given by OSA and  $Mn^{++}$  in the absence of oxalosuccinic carboxylase (Curve 2), constituting merely a quantitative exaggeration of the deviation of Curve 2 from that of the OSA spectrum (Curve 1). This fact clearly indicates that

TABLE I

*Effect of Potassium Chloride on Enzymatic Decarboxylation of Oxalosuccinic Acid*

The Warburg vessels contained 0.021 M citrate buffer of pH 5.6 and 0.007 M oxalosuccinic acid (adjusted with NaOH to pH 5.6), either with or without enzyme, 0.0013 M  $MnCl_2$ , and 0.134 M KCl, as indicated. Final volume 2.8 cc. Air in gas phase. Temperature, 16°. Oxalosuccinate tipped in from side bulb after temperature equilibration.

Additions	CO <sub>2</sub> evolved in 5 min.			
	Pig heart extract (1.6 mg. protein)		"Acetone enzyme" (1.3 mg. protein)	
		Change due to enzyme		Change due to enzyme
	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
$Mn^{++}$ .....	51		33	
Enzyme and $Mn^{++}$ .....	138	87	86	53
$Mn^{++}$ and KCl.....	42			
Enzyme, $Mn^{++}$ , and KCl.....	190	148	114	81

oxalosuccinic carboxylase increases the rate of formation of the OSA-Mn complex.

When  $Mg^{++}$  is substituted for  $Mn^{++}$  at similar concentrations, the addition of oxalosuccinic carboxylase to a mixture of OSA and the cation, whether in the absence or presence of KCl, produces little or no spectrophotometric change beyond that obtained on mixing OSA and  $Mg^{++}$  in the absence of the enzyme. This is in agreement with the observed ineffectiveness of  $Mg^{++}$  in manometric experiments. Since  $Mg^{++}$  and  $Mn^{++}$  bring about similar changes on the absorption of OSA, it would appear that the ineffectiveness of  $Mg^{++}$  with oxalosuccinic carboxylase is due to lack of affinity between the protein and this metal.

Further evidence that the spectrophotometric changes observed on mixing oxalosuccinic carboxylase and OSA, in the presence of  $Mn^{++}$ , are strictly specific and intimately related to the decarboxylation of the keto acid is

furnished by (a) the failure of oxalosuccinic carboxylase to produce any effect on OAA either in spectrophotometric or manometric (1) experiments, and (b) the absence of either spectrophotometric or manometric (1) effects of oxalacetic carboxylase on OSA. All these results indicate that there are two factors involved in the formation of an active oxalosuccinic carboxylase system: the possibility of complex formation between  $\beta$ -keto acid and metal, and the specific affinity of the enzyme for each of these.

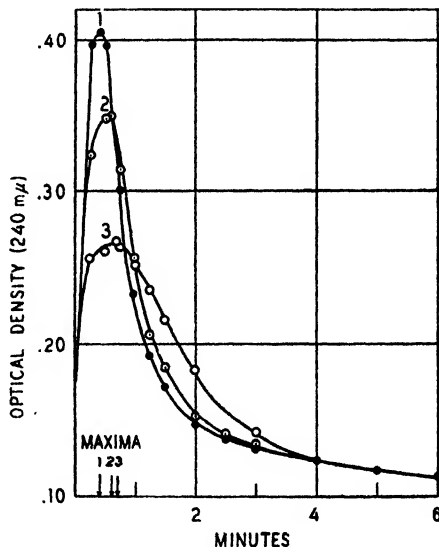


FIG. 5. Spectral changes of oxalosuccinate decarboxylation at various enzyme concentrations. All samples contained  $1.67 \times 10^{-4}$  M oxalosuccinate,  $1.67 \times 10^{-4}$  M  $\text{MnCl}_2$ , and 0.134 M KCl. Total volume of reaction mixtures, 3.0 cc. ( $d = 1.0$  cm.). Wave-length, 240  $\mu$ . Other data as in Fig. 1. Curves 1, 2, and 3, 0.03, 0.02, and 0.01 cc. of enzyme (pig heart extract) respectively. The arrows at the lower left indicate the times at which maximum optical densities were reached in each case.

The curves in Fig. 5 demonstrate that there is some proportionality between the concentration of oxalosuccinic carboxylase and the maximum optical density in the presence of constant amounts of  $\text{Mn}^{++}$  and keto acid. It will be observed, in addition, that the time for attainment of the maximum value is related inversely to the enzyme concentration, and also that the rate of subsequent fall of optical density roughly parallels enzyme concentration. These results suggest a rapid and sensitive optical test for oxalosuccinic carboxylase.

Determination of the optimum concentrations of OSA and  $\text{Mn}^{++}$  was made with a constant amount of enzyme with the maximum density as a measure of activity. Under these conditions the optimum concentration of

TABLE II

Comparative Effectiveness of  $Mg^{++}$  and  $Mn^{++}$  on Oxalacetic Carboxylase of *Micrococcus lysodeikticus*

The Warburg vessels contained 0.025 M acetate buffer, pH 5.0, 0.0095 M oxalacetic acid (adjusted with NaOH to pH 5.0), and 0.5 mg. of enzyme. Other additions as indicated. Final volume 2.0 cc. Air in gas phase. Temperature, 25°.

$MgCl_2$	$MnCl_2$	$CO_2$ evolution*	
		$C.M.M.M.$	Change due to metal
$M$	$M$	$C.M.M.M.$	$C.M.M.M.$
		16	
$5 \times 10^{-4}$		21	5
$1 \times 10^{-3}$		48	32
$4 \times 10^{-3}$		64	48
	$8 \times 10^{-3}$	37	21
	$2 \times 10^{-4}$	70	54
	$5 \times 10^{-4}$	84	68

\* During 5 to 15 minutes after tipping oxalacetate from the side bulb.

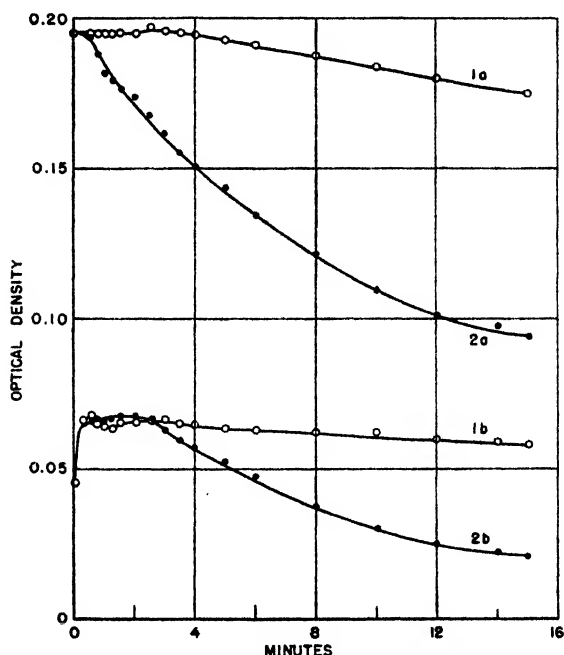


FIG. 6. Spectral changes connected with the enzymatic decarboxylation of oxalacetic acid. Final concentrations,  $2.5 \times 10^{-4}$  M oxalacetate,  $2.5 \times 10^{-4}$  M  $MnCl_2$ . Total volume of reaction mixtures, 2.0 cc. ( $d = 0.5$  cm.). Wave-length, Curves 1a and 2a, 230  $m\mu$ ; Curves 1b and 2b, 290  $m\mu$ . Other data as in Fig. 1. Curves 1a and 1b, oxalacetate and  $MnCl_2$ ; Curves 2a and 2b, oxalacetate,  $MnCl_2$ , and 0.5 mg. of enzyme from *Micrococcus lysodeikticus*.

OSA was about  $2 \times 10^{-4}$  M, while concentrations over  $4 \times 10^{-4}$  M were inhibitory. Optimum concentrations of  $Mn^{++}$  were also reached at about  $2 \times 10^{-4}$  M.

*Oxalacetic Carboxylase*—Krampitz and Werkman reported that  $Mg^{++}$  and  $Mn^{++}$  were approximately equally effective in stimulating the decarboxylation of OAA in the presence of crude lysed preparations of *Micrococcus lysodeikticus* (2). With a partially purified preparation from the same source we found, as in the case of oxalosuccinic carboxylase, that  $Mn^{++}$  is much more effective than  $Mg^{++}$ . This is shown in Table II.

The curves in Fig. 6 show the spectral changes observed on mixing OAA with  $Mn^{++}$ , either with or without oxalacetic carboxylase, at two different wave-lengths, 230 and 290  $m\mu$ . It should be recalled that at 230  $m\mu$ ,  $Mn^{++}$  does not alter the absorption of OAA, whereas at 290  $m\mu$  it produces its maximum absorption increase (Fig. 3, Curves 4 and 5). It is apparent that the two curves representing enzymatic decarboxylation differ markedly. There is a prompt and steady decline at 230  $m\mu$ , while a transitory plateau, indicating formation of a complex, precedes the decline at 290  $m\mu$ . The activity of the oxalacetic enzyme is not enhanced by increased salt concentration.

#### DISCUSSION

Since metal complexes are probably formed with the enol form of keto acids, the non-enzymatic reactions of OSA and OAA studied in this paper might involve the following steps.

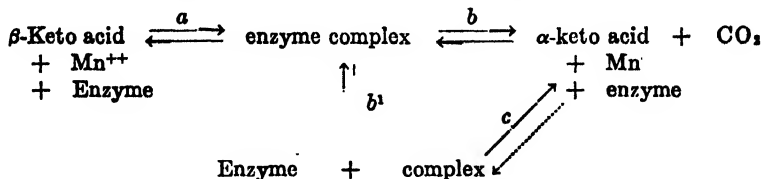
- |       |  |
|-------|--|
| (I)   | $\beta$ -Keto acid $\rightleftharpoons$ enol                       |
| (II)  | Enol + cation $\rightleftharpoons$ complex                         |
| (III) | Complex $\rightleftharpoons$ $\alpha$ -keto acid + $CO_2$ + cation |

None of the above is an instantaneous reaction. Each proceeds at a rate depending upon the nature of both the keto acid and the cation. When the cation is  $Al^{+++}$ , and presumably any which have been shown to accelerate the decarboxylation of the two keto acids, the above set of reactions would proceed rapidly in the absence of an enzyme, since aluminum complexes are either formed rapidly or are very unstable or both.

Enzymatic catalysis of the over-all reaction,  $\beta$ -keto acid  $\rightleftharpoons$   $\alpha$ -keto acid +  $CO_2$ , might be due to acceleration by the enzyme of one of the above reactions (Nos. I, II, or III), of two of them, or of all three. Since the enzymes are active only in the presence of  $Mn^{++}$  and since this cation by itself is not very effective in accelerating decarboxylation, the enzyme might be considered to catalyze Reaction I, *i.e.* the formation of enol. However, since, as previously mentioned, the spectrophotometric changes produced by  $Mn^{++}$  and  $Mg^{++}$  on OSA, in the absence of enzyme, are about the same, it

would seem that the affinity of the keto acid for each of these two cations is approximately equal. This eliminates the possibility that the enzyme merely acts by catalyzing Reaction I, since one would then expect equal effectiveness of  $Mg^{++}$  and  $Mn^{++}$  in the presence of enzyme.

The mechanism of the enzyme-catalyzed reactions may be conveniently discussed with the accompanying scheme as a basis.



In the case of OSA decarboxylation, our data are in agreement with the view that the enzyme catalyzes the formation of an OSA-Mn complex. It is difficult to interpret the increase in optical density caused by the enzyme as due to anything but increased complex formation. While it is clear that this increase in complex formation can account for a major part of the overall catalysis of decarboxylation, it cannot be determined from our data whether the enzyme-OSA-Mn complex dissociates into enzyme and OSA-Mn complex, which then decarboxylates spontaneously (Reactions  $a + b' + c$ ), or whether the over-all reaction proceeds mainly by way of the partial reactions,  $a$  and  $b$ .

The ability of the enzyme to catalyze the reaction in the direction of carboxylation of  $\alpha$ -ketoglutaric acid has been demonstrated by linking the synthesis of the  $\beta$ -keto acid with reduction to isocitrate (7). This reversal is most likely to proceed by Reactions  $b$  and  $a$ , since a non-enzymatic trimolecular reaction to form OSA-Mn complex from  $\alpha$ -ketoglutaric acid,  $\text{CO}_2$ , and  $\text{Mn}^{++}$  is very unlikely.

In the case of oxalacetic carboxylase the time curve with enzyme,  $\text{Mn}^{++}$ , and OAA at  $290 \text{ m}\mu$  (Fig. 6) does not show much increase in complex over that formed by  $\text{Mn}^{++}$  and OAA in the absence of enzyme but it indicates that, in the presence of the enzyme, the complex decarboxylates more rapidly. Comparison with the curves obtained at  $230 \text{ m}\mu$ , at which the OAA-Mn complex has the same absorption coefficient as OAA, shows that the enzyme must increase the rate of complex formation because the curve at  $290 \text{ m}\mu$  (Curve 2b) shows no decrease in density at a point when that at  $230 \text{ m}\mu$  (Curve 2a) indicates that the OAA is already decarboxylated to some extent. It would then seem that, in this case, the reaction is proceeding by way of the partial reactions,  $a$  and  $b$ .

The action of acetoacetic carboxylase seems to involve an entirely different mechanism from that operating in the case of the oxalosuccinic and oxalacetic enzymes. The decarboxylation of AAA is unaffected by metals and,

although complexes can be formed with polyvalent cations, such complexes are stable. It would appear that the nature of the  $\beta$ -keto acid, and especially its degree of stability and its ability to form unstable metal complexes, largely determines the mechanism by which enzymatic decarboxylation is carried out. With the very unstable OSA there are no indications of the need for a prosthetic group besides  $Mn^{++}$ . With the more stable OAA a prosthetic group might well be involved in addition to  $Mn^{++}$ . Recent work suggests a possible function of biotin in some enzymes concerned with OAA and dicarboxylic acid metabolism (8). In the case of AAA, much more stable than the other two  $\beta$ -keto acids, the work of Davies suggests the presence of an as yet unknown prosthetic group in the carboxylase, and no metal is involved (5).

It is well known that amines have a marked catalytic effect on the decarboxylation of  $\beta$ -keto acids; aniline (9) is widely used for their quantitative estimation. This might lead one to believe that amino groups on side chains of a number of proteins might generally catalyze  $\beta$ -keto acid decarboxylation in an unspecific manner. While this seems to be true to some extent (10), it is doubtful whether this unspecific catalysis is of any significance in the biological reactions involving  $\beta$ -keto acids.

### Methods

*Spectrophotometric Measurements*—To each of two quartz cells there were added equal amounts of the various components, with the exception of the keto acids. These were added only to one cell, while the other cell served as control for 100 per cent light transmission. The addition of keto acid was always made last and was immediately preceded by a zero time reading of the optical density of the experimental solution. No buffers were used other than the keto acids themselves, although, as already mentioned, an increase in pH may cause some turbidity in the presence of aluminum ions. Measurements were made in air at room temperature in the Beckman spectrophotometer. Other experimental details are given in the legends to the figures.

*Enzyme Preparations*—The oxalosuccinic carboxylase was a dialyzed extract of washed, acetone-dried pig heart (1) containing from 3 to 5 mg. of protein per cc. In some cases the ice-cold extract was precipitated with 3 volumes of acetone at 0°. An aqueous solution of the dried acetone precipitate, after removal of an insoluble residue by centrifugation, was then used as the enzyme. This is referred to as "acetone enzyme." The oxalacetic carboxylase was a partially purified preparation from *Micrococcus lysodeikticus*.<sup>1</sup> This was kept as a dry acetone powder and was dissolved in water before use.

*Chemical Preparations*—The preparation of oxalosuccinic acid has already

been described (1). The barium salt was dissolved in water, with the aid of some dilute hydrochloric acid, and converted to the sodium salt just before use. Oxalacetic acid was obtained by hydrolysis of diethyl oxalacetate according to Krampitz and Werkman (2), with modifications suggested by Dr. Fritz Lipmann.<sup>5</sup> The preparation of acetoacetic acid was made according to Davies (5). Crystalline sodium pyruvate and  $\alpha$ -ketoglutaric acid were prepared as previously described (11).

#### SUMMARY

The catalytic decarboxylation of oxalosuccinic and oxalacetic acids by aluminum ions is preceded by the formation of unstable intermediates having characteristic ultraviolet absorption spectra. The peaks are at 252 and 274  $m\mu$  respectively. The intermediates are assumed to be  $\beta$ -keto acid-aluminum complexes which undergo rapid decarboxylation. Acetoacetic acid forms a similar but stable complex with  $Al^{+++}$ . Spectral changes occurring with  $Mg^{++}$  and  $Mn^{++}$  also suggest the formation of complexes with these cations.

In the presence of  $Mn^{++}$  and oxalosuccinic acid, oxalosuccinic carboxylase causes a pronounced increase in absorption at 240  $m\mu$ , presumably as a result of increased formation of an intermediate oxalosuccinate-manganese complex; this increase is followed by a rapid drop, indicating decarboxylation. These effects of the enzyme are markedly augmented, as is the decarboxylation of oxalosuccinate followed manometrically, by an increase of the ionic strength of the reaction mixtures to 0.134 M.

In the presence of  $Mn^{++}$  and oxalacetic acid, oxalacetic carboxylase brings about a rapid decrease in absorption, indicating decarboxylation, at 230  $m\mu$ . At 290  $m\mu$  this decrease is preceded by a small transient increase. In this case, if the carboxylase brings about an increased formation of an intermediate oxalacetic-manganese complex, the decarboxylation of this complex must be increased to a similar extent.

The spectrophotometric and manometric changes produced by oxalosuccinic and oxalacetic carboxylases on their respective substrates are strictly specific with regard to  $\beta$ -keto acid and metal.

The above results are discussed in connection with the mechanism of the reactions catalyzed by cations and by  $\beta$ -keto acid carboxylases.

We wish to thank Dr. Isidor Greenwald and Dr. Milton Levy for helpful suggestions. We are also indebted to Mr. Morton C. Schneider for skilful technical assistance.

<sup>5</sup> Personal communication. We are very indebted to Dr. Lipmann for this information.



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THE STEREOCHEMISTRY OF AN ENZYMATIC REACTION:  
THE OXIDATION OF *l*-, *d*-, AND *epi*-INOSITOL  
BY ACETOBACTER SUBOXYDANS\*

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The property of certain members of the group of acetic acid bacteria (1) that brings about the incomplete oxidation of various substrates, stopping often at the primary oxidation product, is emphasized in the name of the bacterial type with which this paper deals, *Acetobacter suboxydans* (2). The action of this organism on polyhydroxy compounds terminates, as a rule, with the formation of a monoketo compound. Certain sterical limitations, observed experimentally, have found their expression in the well known rule of Bertrand (3) which, however, does not apply to all substrates (1, 4).

It occurred to us that the stereochemical specificity of this interesting enzyme system could be defined with greater precision if a stereoisomeric series of cyclic polyhydroxy compounds were employed as the substrates of oxidation, since in this case the lack of free rotation around carbon to carbon bonds should permit a clearer correlation between reactivity and the spatial arrangement of the reactive groups. For this purpose, several isomers of the inositol group and related cyclitols were chosen. The oxidation rates were studied manometrically and whenever possible the oxidation products isolated and identified.

The experiments, which in part have been presented very briefly in a preliminary communication (5), were carried out with resting cells.<sup>1</sup> Since the localization of the enzyme system within the bacterial cell is unknown, it is not yet possible to present an adequate discussion of the reasons for the differences in oxidation rates found for the several stereoisomers. Should the speed of permeation of the substrate into the cell prove to be the rate-determining step, then it would become necessary to consider the bacterial

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† This report is from a dissertation to be submitted by Boris Magasanik in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

<sup>1</sup> The oxidative enzymes of *Acetobacter* have so far not been obtained in a cell-free state (1).

cell wall as a sterically selective system. If the enzyme is situated at the bacterial surface or if a non-selective diffusion operates, the rates of oxidation would be an expression of the equilibrium conditions governing the formation of the particular substrate-enzyme complex or its reaction with oxygen. (For recent discussions of permeability, cf. (6, 7).) In any event, the selection for enzymatic attack of one or more of the sterically distinct secondary hydroxyl groups of the inositol must derive from the direct action of the bacterial oxidase on the cyclitol. The nature of the *Acetobacter* oxidase or oxidases, however, still is completely unknown.

While it cannot yet be stated definitely that the same enzyme is responsible for the oxidation of the different stereoisomers discussed here, the preliminary information available would appear to favor this assumption. Mixtures of *meso*- and *d*-inositol, each in a concentration sufficient to saturate the enzyme system, were found to be oxidized at a rate identical with that at which either of the components alone was handled. Furthermore, the resting bacteria (cultivated in the presence of D-sorbitol, but in the absence of the inositol isomers) oxidized even as unusual substrates as *epi*-inositol or *d-epi*-inosose without a lag phase, as should have been the case if adaptation had been a requisite for enzymatic oxidation.

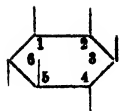
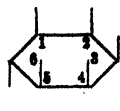
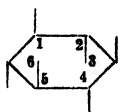
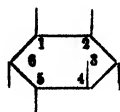
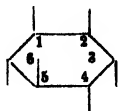
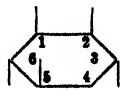
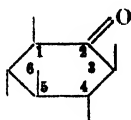
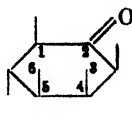
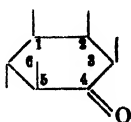
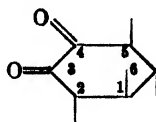
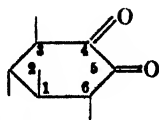
The planar projections of the cyclitols used as substrates and of some of the oxidation products are shown in structures I to XI. The structure of I is based on the work of Dangschat and Fischer (8) and of Posternak (9), and that of II (10), III (9), IV (11), VI (12), and VII (9) on the series of brilliant studies by Posternak.<sup>2</sup>

The *maximal oxygen consumption* of the various substrates is summarized in Table I. In these experiments, carried out with a high concentration of bacteria, the diffusion of oxygen into the suspension became the limiting factor. Veronal buffer could be substituted for the usually employed phosphate buffer of pH 6.0 without changing the nature of the oxidation of *meso*-inositol.

In the case of *meso*-inositol (I) the results of Kluyver and Boezaardt (14) were confirmed. The consumption of 1 gm. atom of oxygen per mole of substrate indicated the formation of a monoketo derivative, *meso*-inosose (VII). This compound has frequently been isolated in different laboratories (14-16).

Similar results were obtained with *epi*-inositol (II) which yielded a levoro-

<sup>2</sup> The numbering of the carbon atoms is similar to the one adopted by Ebel ((13) p. 619). The carbon atoms are numbered clockwise, 1 and 6 chosen with their hydroxyls in trans position. The structure is arranged in such a manner as to have as many hydroxyl groups as possible cis to the one in position 1. Where this scheme allows two possibilities, that arrangement is chosen in which the carbon atoms with hydroxyls cis to the one in position 1 have the lowest positional numbers. The same system is used for keto and desoxy derivatives.

I. *meso*-Inositol; cyclohexane-(1,2,3,5)-*cis*-hexolII. *epi*-Inositol; cyclohexane-(1,2,3,4,5)-*cis*-hexolIII. Scyllitol; cyclohexane-(1,3,5)-*cis*-hexolIV. *L*-Inositol; cyclohexane-(1,2,4)-*cis*-hexolV. *d*-Inositol; cyclohexane-(1,2,5)-*cis*-hexolVI. *d*-Quercitol; cyclohexane-(1,2,5)-*cis*-3,6-pentolVII. *meso*-Inosose; cyclohexane-(1,3,5)-*cis*-pentol-2-oneVIII. *L-epi*-Inosose; cyclohexane-(1,3,4,5)*cis*-pentol-2-oneIX. *d-epi*-Inosose; cyclohexane-(1,2,3,5)-*cis*-pentol-4-oneX. Cyclohexane-(1,5)*cis*-tetrol-3,4-dioneXI. Cyclohexane-(1,3)*cis*-tetrol-4,5-dione

tatory monoketo compound (5). The study of this derivative, included in the experimental part, served to establish its structure as either VIII or IX, that of *epi*-inositol as II, and that of the inosose obtained by the action of

nitric acid on *meso*-inositol (17) as the *dl* compound VIII + IX; but since analogous findings were in the meantime presented independently by Posternak (10), who proved the structure of the levorotatory ketone to be VIII, a detailed discussion appears superfluous.

*dl*-*epi*-Inosose (VIII + IX), formed by the action of nitric acid on I, was found to consume 0.25 mole of oxygen per mole of substrate. Inasmuch as *l*-*epi*-inosose (VIII), the end-product in the oxidation of *epi*-inositol, is re-

TABLE I  
*Maximal Oxygen Consumption*

The Warburg vessels in each experiment contained 0.5 cc. of the suspension of resting *Acetobacter suboxydans* (about 5 mg. of bacterial N) and 2 cc. of 1/15 M phosphate buffer of pH 6.0 in the main compartment, 0.5 cc. of the substrate solution in the side arm, and 0.2 cc. of 10 per cent KOH in the center well. The experiments were carried out at 38° in the presence of air.

Substrate		Oxygen consumption, moles per mole substrate	Duration of oxygen uptake
	<i>micromoles</i>		<i>min.</i>
<i>meso</i> -Inositol	6.6	0.47, 0.50	10
	13.9	0.50, 0.50	15
	13.9*	0.51, 0.47	15
<i>l</i> -Inositol	6.6	1.01, 1.03, 1.00	20-25
<i>d</i> -Inositol	10.0	1.00, 0.97, 0.99	50
Scyllitol	5.8	0	
<i>epi</i> -Inositol	8.7	0.51, 0.49	10
<i>dl</i> - <i>epi</i> -Inosose	6.8	0.25	15
	13.4	0.27, 0.27	15
	17.7	0.25, 0.26	20
Quebrachitol	12.5	0	
Pinitol	7.8	0	
<i>d</i> -Quercitol	7.0	0.95, 0.97, 0.96, 1.01	50†
	7.0‡	0.50, 0.47	35

\* In this experiment veronal buffer (pH 6.0) was substituted.

† The first gm. atom of oxygen was consumed within about 7 minutes.

‡ This experiment was performed with only about 0.7 mg. of bacterial N per vessel.

sistant to further oxidation, one may conclude that each mole of *d*-*epi*-inosose (IX) reacted with 0.5 mole of oxygen, giving rise to a diketone compound. Attempts at the isolation of this derivative have not yet been successful. The second carbonyl group probably is not vicinal to the one present in IX, since such  $\alpha$ -diketones readily give rise to bisphenylhydrazones; it more probably occupies position 2 of IX, by analogy to the oxidation of *epi*-inositol. The oxidation product was found to have acidic properties, which is in agreement with the expected behavior of an enolized  $\beta$ -diketone.

The two optically active stereoisomers, *l*-inositol (IV) and *d*-inositol (V), each reacted with 2 gm. atoms of oxygen per mole of substrate. From 5 to 20 day-old culture fluids of *Acetobacter suboxydans* containing these substances the oxidation products could be isolated by means of phenylhydrazine, but only in the case of the *l* compound was the yield good. Both products obtained in this manner from *l*- and *d*-inositol respectively proved to be bisphenylhydrazones of diketo inositols; they formed yellow needles

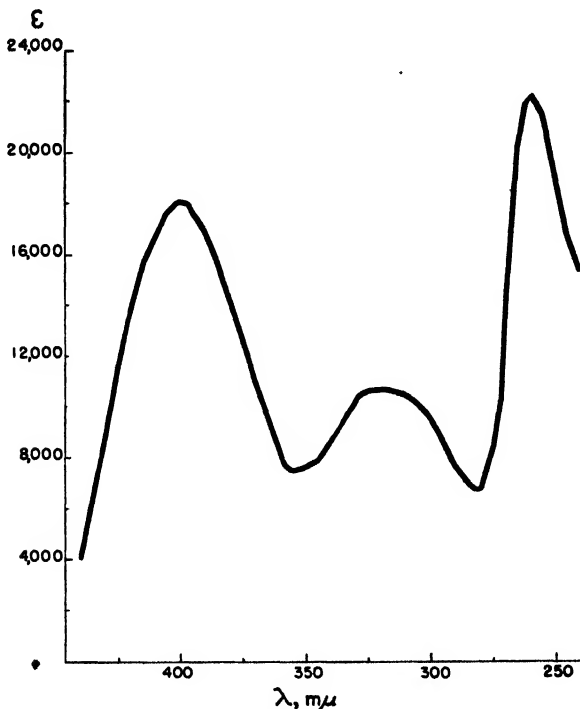
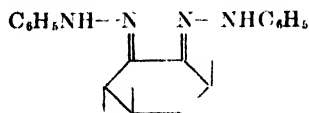


FIG. 1. Absorption spectrum (in absolute ethanol) of the  $\alpha$ -bisphenylhydrazone of cyclohexane-(1,5)*cis*-tetrol-3,4-dione (XII) obtained from *l*-inositol.

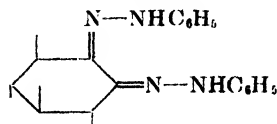
with identical melting points, identical absorption spectra (Fig. 1) characteristic for osazones (18), and optical rotations equal but opposite in sign. When equal amounts of the two substances were permitted to crystallize together, a new compound with a lower melting point, but an unchanged absorption spectrum, was obtained. The two bisphenylhydrazones clearly were enantiomorphs. The consumption of periodic acid in 66 per cent alcohol at room temperature corresponded to 3 moles of the oxidant per mole of bisphenylhydrazone (19). These findings serve to establish these compounds as  $\alpha$ -bisphenylhydrazones. The reaction products obtained by the

action of periodic acid on these substances are discussed in another communication (20).

The exact configurations of the  $\alpha$ -bisphenylhydrazones obtained from the biological oxidation products of *l*- and *d*-inositol were determined by comparing their racemic mixture with an  $\alpha$ -bisphenylhydrazone of a diketo inositol obtained by Carter *et al.* (21) by the action of phenylhydrazine on the phenylhydrazone of *meso*-inosose (VII).<sup>3</sup> In this compound the second phenylhydrazono group must have been introduced into positions 1 and 3 respectively, leading to a racemic mixture of XII and XIII.



(XII)



(XIII)

Both racemic mixtures exhibited the same absorption spectrum, identical with the one shown in Fig. 1, and identical melting points. Their mixture showed no depression of the melting point. Structure XII can, therefore, be assigned to the dextrorotatory compound obtained from *l*-inositol, structure XIII to the levorotatory substance produced from *d*-inositol.

*l*-Inositol (IV) has thus been shown to be oxidized by *Acetobacter suboxydans* in positions 1 and 6 to yield the  $\alpha$ -diketone X; *d*-inositol (V), attacked in the corresponding mirror image positions 2 and 3, affords the  $\alpha$ -diketone XI. This appears to be the first instance of the production by this microorganism of a dicarbonyl compound from a polyhydric alcohol.<sup>4</sup>

*Quebrachitol* and *pinitol*, the naturally occurring monomethyl ethers of *l*- and *d*-inositol respectively, were not oxidized by *Acetobacter*. The position of the methoxyls in these compounds is not known. *Scyllitol* (III) likewise was not attacked.

*d*-*Quercitol* (VI) consumed 2 gm. atoms of oxygen per mole of substrate. The 2nd gm. atom was taken up at a much slower rate. With a lower concentration of bacteria, the oxygen uptake terminated with the consumption of the first half mole, a phenomenon not observed with the optically active inositols.

The rates of oxygen uptake by equal quantities of the various substrates,

<sup>3</sup> We should like to thank Dr. H. E. Carter of the University of Illinois for a specimen of this compound and for a personal communication concerning the mode of formation of this osazone.

<sup>4</sup> The production by *Acetobacter suboxydans* of a diketo derivative from *meso*-inositol has been reported by one group of workers (22-25). This product does not appear to have been observed in other laboratories (14-16) nor is its formation compatible with the manometric measurements presented previously (5, 14) and in this paper.

insufficient in concentration to give maximal oxidation rates, are presented in Table II. When, as is done in Table II, these rates are related to the rate of oxygen consumption by *meso*-inositol at the same bacterial concentration, it will be seen that under the experimental conditions the various substrates were oxidized at widely differing rates, *d*-inositol being the most slowly and *epi*-inositol the most rapidly oxidized stereoisomer. The slow oxidation of *d*-inositol may be responsible for the low yield of oxidation product obtained in the culture experiments.<sup>5</sup>

TABLE II  
*Comparison of Oxidation Rates*

The Warburg vessels in each experiment (carried out at 38°) contained 0.5 cc. of the suspension of resting bacteria and 2 cc. of 1/15 M phosphate buffer of pH 6.0 in the main compartment, 0.5 cc. of solution containing 7 micromoles of substrate in the side arm, and 0.2 cc. of 10 per cent KOH in the center well.

Bacterial N	Substrate	QO <sub>2</sub> (N)	$\frac{QO_2 (N) \text{ (substrate)}}{QO_2 (N) \text{ (meso-inositol)}}$
mg.			
0.36	<i>meso</i> -Inositol	-370	
	<i>epi</i> -Inositol	-620	1.7
0.59	<i>meso</i> -Inositol	-530	
	<i>epi</i> -Inositol	-880	1.7
	<i>d-epi</i> -Inosose*	-280	0.5
0.72	<i>d-epi</i> -Inosose*	-230	0.4†
	<i>d</i> -Quercitol	-240	0.4†
0.89	<i>d-epi</i> -Inosose*	-330	0.5†
0.94	<i>meso</i> -Inositol	-740	
	<i>l</i> -Inositol	-430	0.6
	<i>d</i> -Inositol	-170	0.2

\* 14.0 micromoles of *dl-epi*-inosose were used.

† The corresponding values for *meso*-inositol were interpolated.

The maximal rates of oxidation of *meso*- and *d*-inositol are compared in Table III. In these experiments the enzyme system was saturated with respect to substrate. The maximal rates of oxygen uptake were found identical for both isomers and also for their equimolar mixture (Table III, Preparation 2). But the *meso* compound was oxidized at the maximal rate at a concentration at which the oxidation of *d*-inositol had not yet reached its maximum (Table III, Preparation 1). Some of the possible explanations of this difference in saturation levels have been mentioned above. If the influence of the cell wall on the speed of diffusion of the two isomers is dis-

<sup>5</sup> A much better yield of derivative XIII could be obtained in preparatory experiments with resting bacteria, as will be described on a later occasion.



regarded, the results shown in Table III seem to indicate that the equilibrium conditions governing the combination between enzyme and substrate are responsible for the differences.

The steric specificity of the enzyme system responsible for the oxidations that form the subject of this paper can now be discussed succinctly. It is, of course, evident that the structural formulae I to XIII of this series of cyclohexane derivatives do not describe the actual positions in space of the various atoms, but merely represent planar projections based on the conventions introduced into stereochemistry by Emil Fischer (26).

TABLE III  
*Maximal Rates of Oxidation*

The Warburg vessels in each experiment (38°) contained 0.5 cc. of the bacterial suspension and 2 cc. of 1/15 M phosphate buffer of pH 6.0 in the main compartment, 0.5 cc. of substrate solution in the side arm, and 0.2 cc. of 10 per cent KOH in the center well.

Bacterial preparation No.	Bacterial N	<i>meso</i> -Inositol	<i>d</i> -Inositol	$Q_{O_2}(N)$	$\frac{Q_{O_2}(N) \text{ (substrate)}}{Q_{O_2}(N) \text{ (meso-inositol)}}$
	mg.	micromoles	micromoles		
1	0.47	28.2		-990	
		56.4		-1100	
			28.2	-340	0.35
			56.4	-680	0.62
			28.2	-940	0.85
2	0.15	28.2		-1700	
		225		-1570	
		450		-1730	1.0
			225	-1920	1.2
			450	-1680	1.1

Recent work based on electron diffraction studies (27) and spectroscopic (28) and thermodynamic (29) properties has led to the conclusion that at room temperature cyclohexane exists predominantly in the chair configuration. If the conceptions applied to cyclohexane and its mono- and dimethyl derivatives (29, 30) are extended to the inositols, it will be seen that six of the substituents (hydroxyl or hydrogen) surround the ring of carbon atoms in what has been called an equatorial belt; the six others are perpendicular to the plane formed by the carbon ring: three above (north polar), three below (south polar). Each carbon atom will, therefore, carry 1 polar and 1 equatorial substituent. A twist of the 6 carbon atoms through a single plane to the opposite chair form renders the equatorial substituents polar and vice versa (30).

Consequently, there will exist two geometrically tautomeric forms for each inositol isomer. By analogy to the methyl-substituted cyclohexanes (29) it may, however, be assumed that the tautomer possessing the smaller number of polar hydroxyls will predominate. The constellations of compounds I to XI, based on the postulates outlined here, are compared in Table IV.

The inspection of atomic models of the inositols shows the position of the hydroxyl groups to be very unfavorable to the formation of cyclic compounds, such as benzyldene or isopropylidene derivatives. This is in line

TABLE IV  
*Spatial Configurations of Inositol Isomers and Related Substances*

Planar projection	Compound	Position of hydroxyls on carbon atoms			Position of hydroxyls oxidized by <i>Acetobacter suboxydans</i>
		Equatorial	North polar	South polar	
I	<i>meso</i> -Inositol	1, 3, 4, 5, 6	2	—	2
II	<i>epi</i> -Inositol	1, 3, 5, 6	2, 4	—	2
III	Scyllitol	1, 2, 3, 4, 5, 6	—	—	—
IV	<i>l</i> -Inositol	2, 3, 4, 5	1	6	1, 6
V	<i>d</i> -Inositol	1, 4, 5, 6	2	3	2, 3
VI	<i>d</i> -Quercitol	1, 5, 6	2	3	*
VII	<i>meso</i> -Inosose	1, 3, 4, 5, 6	—	—	—
VIII	<i>l-epi</i> -Inosose	1, 3, 5, 6	4	—	—
IX	<i>d-epi</i> -Inosose	1, 3, 5, 6	2	—	†
X	Cyclohexane - (1,5) <i>cis</i> - tetrol-3,4-dione	1, 2, 5, 6	—	—	—
XI	Cyclohexane - (1,3) <i>cis</i> - tetrol-4,5-dione	1, 2, 3, 6	—	—	—

\* 2 gm. atoms of oxygen taken up per mole of substrate.

† 1 gm. atom of oxygen taken up per mole of substrate.

with the great difficulty with which the condensation of *meso*-inositol with acetone is brought about (8).

Recent work on the configuration and the biological activity of the hexachlorocyclohexanes also provides interesting analogies to the inositols. Among the structures, assigned by Slade (31) to the different isomers,  $\gamma$ -hexachlorocyclohexane corresponds to configuration I in Table IV, the  $\alpha$  isomer to a racemic mixture of IV and V, and the  $\beta$  compound to III. The biological antagonism of  $\gamma$ -hexachlorocyclohexane to *meso*-inositol as a growth factor for yeast (32) also is suggestive of a spatial similarity between these compounds. Moreover, the melting points of the two series of cyclohexane derivatives show a parallel trend. *meso*-Inositol, *dl*-inositol, and scyllitol melt at 218°, 253°, and 345° respectively; the respective melting

points of the  $\gamma$ ,  $\alpha$ , and  $\beta$  isomers of hexachlorocyclohexane are at  $112^\circ$ ,  $158^\circ$ , and  $309^\circ$ .

The specific steric requirements for oxidation can, on the basis of the structures assigned to the inositols (Table IV), be expressed as follows: *Only polar hydroxyl groups are oxidized.* In the case of *epi*-inositol (II), where two hydroxyls are situated in the same polar plane, only one is attacked by the enzyme to yield *l-epi*-inosose (VIII). The enantiomorph *d-epi*-inosose (IX), however, is further oxidized. Additional work will be necessary to show whether the relative position of the polar hydroxyls with respect to the hydrogen atom in the same polar plane is of importance for the extent of oxidation.

The study of the oxidation of aliphatic polyhydric alcohols by *Acetobacter* has led to the conclusion that only those hydroxyl groups are attacked that are situated between a primary hydroxyl and a secondary one in *cis* position (3, 4). Of the enantiomorphs, the isomer possessing the *D* configuration with respect to the secondary *cis* hydroxyls is oxidized more readily (4). A similar relationship appears to apply to cyclic enantiomorphs of the inositol series. In *l*-inositol, which is much more rapidly oxidized than the *d* isomer, the hydroxyl groups that are oxidized and the neighboring *cis* hydroxyl groups have the *D* configuration.

The findings reported here permit the inference that, in the aliphatic polyhydroxy compounds attacked by the *Acetobacter*-oxidase system, the position in space of a secondary hydroxyl group between a primary alcohol group and another secondary *cis* hydroxyl corresponds to the position of a polar hydroxyl group between two equatorial ones in the inositol molecule.

It should be pointed out that the studies on the enzymatic oxidation of inositol isomers, which are being continued, may prove of more general biological interest in several respects. The great importance of *meso*-inositol as a growth factor for certain microorganisms ((33) p. 207), the discovery of a deficient mutant of *Neurospora crassa* requiring inositol (34), and the dietary significance of this compound in animal nutrition (35) all require a better insight into the ways in which *meso*-inositol and its isomers are dealt with by living cells. Moreover, one of the constituents of streptomycin has been shown to be a 1,3-diguanido-2,4,5,6-tetrahydroxycyclohexane (36, 37). In view of what has been said above about the oxidation of *d-epi*-inosose (IX) by *Acetobacter*, it is possible that additional work will lead to the preparation of 1,3-substituted inositol derivatives of biological interest.

## EXPERIMENTAL

### Material

*Acetobacter suboxydans* was obtained from the American Type Culture

Collection (No. 621). The culture methods employed will be discussed below.

*d*-Quercitol and *meso*-inositol were purified commercial preparations.

A crude preparation of *pinitol* was obtained through the courtesy of Professor R. C. Elderfield of this University. It was purified by repeated decolorization of its aqueous solution with charcoal (Darco G-60) and crystallization from 75 per cent ethanol. The pure *pinitol*, melting<sup>6</sup> at 185–186°,  $[\alpha]_D^{26} = +66.4^\circ$ , yielded, on treatment with hydriodic acid (38), *d*-inositol (86 per cent of the theoretical yield, crystallized from 72 per cent ethanol) which had a melting point of 249–250° and  $[\alpha]_D^{25} = +65.0^\circ$  (in water).

The crude *quebrachitol*, obtained some years ago from the British Rubber Research Association, London, was freed of traces of sulfate by treatment with barium hydroxide and crystallized by the addition of 1.8 parts of hot acetone to the hot aqueous solution. The pure monomethyl ether melted at 195°. It yielded, when treated with hydriodic acid, *l*-inositol (83 per cent of the theory, crystallized from 70 per cent ethanol) which melted at 249–250° and had  $[\alpha]_D^{22} = -63.8^\circ$  (in water).

*Syllitol*<sup>7</sup> was prepared from *meso*-inositol by the method of Posternak (15), m.p. 345° (with decomposition); its hexaacetyl derivative melted at 296–297°. *dl*-*epi*-Inosose, m.p. 221° (with decomposition), was prepared by the action of nitric acid on *meso*-inositol (17). *epi*-Inositol, m.p. 285° (with decomposition), was obtained by the catalytic hydrogenation of *dl*-*epi*-inosose (17).

#### *Oxidation of Inositol Stereoisomers and Related Substances by Acetobacter suboxydans*

*Cultivation of Bacteria*—The cells were grown at 30° in a medium containing 50 gm. of D-sorbitol and 10 gm. of yeast extract (Difco) in 1 liter of tap water. When larger quantities of resting organisms were required, the cultivation was carried out in Roux bottles containing 150 cc. of the liquid medium and 4.5 gm. of agar. A 3 cc. portion of a 3 day-old liquid culture served as the inoculum for each Roux bottle. After 70 hours the cells were collected in physiological saline, centrifuged in the cold at 4000 r.p.m., and washed three times with ice-cold saline. They were then again suspended in physiological saline. In all experiments bacteria collected the same day were used.

<sup>6</sup> The melting points, reported without correction, were determined with an electrically heated stage (Fisher-Johns). Unless noted otherwise, the intact crystals were placed on the stage preheated to about 5–7° below the melting point.

<sup>7</sup> The melting points of *scyllitol* and hexaacetyl *scyllitol* were determined in capillaries.

**Maximal Oxygen Consumption**—When 0.5 cc. of a bacterial suspension in physiological saline containing about 5 mg. of bacterial nitrogen was permitted to act on 5 to 18 micromoles of the various substrates in a total volume of 3 cc., very rapid oxidation took place. The reaction was considered as terminated when the amount of oxygen consumed in a 5 minute interval was no greater than that taken up in the same period by the resting bacteria alone (i.e. about 2 microliters). Table I summarizes the results.

**Comparison of Oxidation Rates**—The rates at which the several substrates were dehydrogenated were compared in a series of experiments in each of which 7 micromoles of substrate and small amounts of resting bacteria (0.36 to 0.94 mg. of bacterial nitrogen) were employed. These experiments are presented in Table II. For the calculation of the  $Q_{O_2}$  (N), the time interval for which the oxygen uptake was linear was used.

**Maximal Rates of Oxidation**—In the preceding experiments the enzyme system was not saturated with respect to substrate. The saturation levels for *meso*- and *d*-inositol were compared in another experiment reproduced in Table III.

#### *Formation of Compound X from l-Inositol*

**Preparation**—*Acetobacter suboxydans* was grown for 3 days at 30° in a medium consisting of 0.2 gm. of D-sorbitol, 0.2 gm. of yeast extract (Difco), and 0.5 gm. of *l*-inositol in 10 cc. of tap water. The culture was then transferred under sterile conditions to a 2.5 liter Fernbach flask containing a sterilized solution of 4.5 gm. of *l*-inositol in 90 cc. of tap water. Following incubation for 5 days at 30° the bacteria were removed by filtration through infusorial earth and a solution of 10 cc. of freshly distilled phenylhydrazine in 20 cc. of 50 per cent acetic acid was added to the filtrate. Crystals soon made their appearance at room temperature and transformed the mixture which was cooled overnight to a semisolid paste. The crystals which at this stage were contaminated with a red oil were filtered off, washed with ice-cold water, and spread on a porous plate. To their filtered solution in 75 cc. of hot pyridine, 200 cc. of hot water were added slowly. The chilled mixture deposited 4.2 gm. of reddish yellow crystals (42 per cent of the theoretical yield) which were recrystallized from a 1:1.6 mixture of water and methyl cellosolve (ethylene glycol monomethyl ether), following the treatment of the methyl cellosolve solution with 5 gm. of activated charcoal (Darco G-60). The crystals (2.9 gm.) were recrystallized once more from 3 parts of methyl cellosolve and 2 parts of water when 2.6 gm. of the  $\alpha$ -bisphenylhydrazone of cyclohexane-(1,5)*cis*-tetrol-3,4-dione (XII) were obtained as long yellow needles with a greenish sheen melting (with decomposition) at 217°. The optical rotation in 1:1 ethanol-pyridine ( $c = 0.793$ ,  $l = 0.5$  dm.,  $\alpha_D = +0.95^\circ$ ) was found as  $[\alpha]_D^{25} = +240^\circ$  (initial); after 24 hours ( $\alpha_D = +0.85^\circ$ ) it was  $[\alpha]_D^{25} = +214^\circ$ .

$C_{12}H_{20}O_4N_4$  (356.4). Calculated. C 60.7, H 5.7, N 15.7  
 Found. " 60.8, " 5.8, " (Dumas) 15.4

The absorption spectrum of this phenylosazone (0.0273 mm solution in absolute ethanol) is reproduced in Fig. 1.

The substance was extremely refractory to attempts at splitting or conversion. Efforts to prepare the free diketone by the treatment of the osazone with pyruvic acid (39) led to no result, nor was it possible to obtain the corresponding osotriazole (40).

*Action of Periodic Acid on Compound XII*—The  $HIO_4$  consumption was determined by treating 20 cc. portions (each containing 36.5 micromoles of the osazone in 60 per cent ethyl alcohol) with 325 micromoles of periodic acid in 0.5 cc. of water at room temperature for 1 hour (19). The excess periodic acid was determined with 0.1 N sodium arsenite in the usual manner. The average consumption (per mole of compound) of periodic acid was 3.05 moles.

#### *Formation of Compound XI from d-Inositol*

10 cc. of a 3 day-old culture of *Acetobacter suboxydans* containing 2 per cent D-sorbitol, 2 per cent yeast extract (Difco), and 5 per cent *d*-inositol were added to a sterile solution of 9.5 gm. of *d*-inositol in 140 cc. of tap water. After incubation at 30° for 14 days the mixture, which showed heavy growth, was filtered through infusorial earth. The red crystals, appearing soon after the addition to the filtrate of 25 cc. of phenylhydrazine in 50 cc. of 50 per cent acetic acid, were washed with ethyl alcohol and dried.<sup>8</sup> The recrystallization of this material (4.7 gm.) from a mixture of 2 parts of water and 3 parts of methyl cellosolve yielded 260 mg. of the  $\alpha$ -bisphenylhydrazone of cyclohexane-(1,3)cis-tetrol-4,5-dione (XIII). The long yellow needles, after one additional recrystallization from aqueous methyl cellosolve melted (with decomposition) at 217°. The optical rotation in 1:1 ethanol-pyridine ( $c = 0.567$ ,  $l = 0.5$  dm.,  $\alpha_D = -0.71^\circ$ ) was found as  $[\alpha]_D^{25} = -250^\circ$ ; at equilibrium (after 24 hours)  $\alpha_D$  was  $-0.64^\circ$ ; therefore  $[\alpha]_D^{25} = -222^\circ$ .

$C_{12}H_{20}O_4N_4$  (356.4). Calculated. C 60.7, H 5.7, N 15.7  
 Found. " 61.0, " 5.7, " (Dumas) 15.3

The absorption spectrum of the levorotatory bisphenylhydrazone corre-

<sup>8</sup> The crude crystals were found to contain, in addition to the osazone, a phenylhydrazone of a monoketo derivative of *d*-inositol which could be separated by virtue of its complete insolubility in hot absolute ethanol. Several recrystallizations from aqueous pyridine yielded colorless plates which melted (with decomposition) at 197–199°. Calculated for  $C_{12}H_{18}O_4N_2$  (268.3), C 53.7, H 6.0, N 10.4; found, C 53.3, H 5.7, N 10.0.

sponded completely with that exhibited by the dextrorotatory derivative (Fig. 1) obtained from *l*-inositol.

This substance, when examined as described before in this paper, was found to consume 2.9 moles of periodic acid.

### *Racemic Inosazone*

A mixture of equal amounts of the *d*- and *l*-inosazones, described in the preceding sections, was recrystallized from 2:3 water-methyl cellosolve. The yellow crystals of the racemic mixture melted (with decomposition) at 205° when placed on the stage at 200°.

The properties of these racemic crystals were compared with those of the phenylosazone obtained by the action of phenylhydrazine on the phenylhydrazone of *meso*-inosose (21). This compound, recrystallized from 2:3 water-methyl cellosolve, likewise exhibited a melting point (with decomposition) of 205° (stage preheated to 200°). Both substances gave the same absorption spectrum, which was identical with the one reproduced in Fig. 1.

For the determination of mixed melting points, the effect of crushing on the melting points of the various substances had to be studied. The finely powdered preparations were found to decompose, without truly melting, at temperatures somewhat lower than the melting points of the crystals themselves. The powders of the dextrorotatory (XII) and levorotatory (XIII) osazones both had decomposition points of 210°; their mechanical mixture decomposed at 203–206°. The finely powdered racemic mixture obtained by joint crystallization of the two osazones decomposed at 199°, the osazone described by Carter *et al.* (21) at 198–199°. No depression of the decomposition point was observed with a mixture of the two racemic osazones.

### *Formation of l-epi-Inosose (VIII) from epi-Inositol (II)*

5 cc. of a 3 day-old culture of *Acetobacter suboxydans* in the sorbitol-yeast extract medium were added to a sterile solution of 5 gm. of *epi*-inositol, 0.85 gm. of yeast extract (Difco), and 0.17 gm. of *D*-sorbitol in 170 cc. of tap water. The mixture was incubated in a large Fernbach flask at 30° for 7 days. The culture fluid, which showed heavy growth, was filtered through infusorial earth and the filtrate concentrated to dryness *in vacuo*. The solution of the residue in 10 cc. of water deposited 3.0 gm. of crystalline material on the addition of 90 cc. of ethanol. The crude product was dissolved in 15 cc. of water and converted to the phenylhydrazone by the addition of 3 cc. of phenylhydrazine in 6 cc. of 50 per cent acetic acid.

The phenylhydrazone was washed with water and ethyl alcohol (weight 3.17 gm.; decomposition point 197°) and decomposed in the customary manner by refluxing its suspension in 65 cc. of water with 4.8 cc. of freshly distilled benzaldehyde and a small amount of benzoic acid for 20 minutes.

The filtrate from the chilled reaction mixture was extracted with ether and evaporated to dryness *in vacuo*. To the solution of the residue in 25 cc. of hot water 100 cc. of hot ethanol were added. The mixture, following filtration while hot, deposited, on being chilled, 524 mg. of *l-epi-inosose* (VIII), white needles melting (with decomposition) at 194–196°. Another preparation of this ketone, obtained in a similar manner, melted (with decomposition) at 198°. The substance was levorotatory in water ( $c = 2.54$ ,  $l = 2$  dm.,  $\alpha_D = -0.26^\circ \pm 0.02^\circ$ );  $[\alpha]_D^{27} = -5.1^\circ \pm 0.4^\circ$ . It showed no mutarotation. Its solution reduced Benedict's solution in the cold.

$C_6H_{10}O_6$  (178.1). Calculated, C 40.5, H 5.7; found, C 40.4, H 5.7

For reduction, 50 mg. of *l-epi-inosose* in 6 cc. of water were shaken with four 300 mg. portions of 2.5 per cent sodium amalgam. The reaction mixture was kept acid by the addition of four portions of 0.37 cc. of N acetic acid. The dry evaporation residue of the supernatant was heated under a reflux with 2 cc. of acetic anhydride for 20 minutes. The excess reagent was decomposed with 5 volumes of water. The chilled mixture deposited 77 mg. of crystalline material which was extracted with 0.8 cc. of boiling ethanol. The insoluble residue was recrystallized from 6 cc. of absolute ethanol, when 22 mg. of *hexaacetyl meso-inositol* were obtained, melting at 216–217°. Admixture of an authentic specimen of this hexaacetate produced no depression of the melting point.

The authors are indebted to Mr. W. Saschek for the microanalyses.

#### SUMMARY

The mechanisms of oxidation by *Acetobacter suboxydans* of several isomers of the inositol group and related cyclitols have been studied. The extent and rate of oxygen uptake by the different substrates have been determined and the oxidation products or their derivatives isolated and identified. *epi-Inositol* has been shown to yield a monoketone, *l-epi-inosose*; *l-* and *d-inositol* gave rise to  $\alpha$ -diketones whose configurations have been ascertained. Scyllitol and the monomethyl ethers quebrachitol and pinitol were not attacked. On the basis of these results and in the light of recent work on the stereochemistry of cyclohexane, the minimum steric requirements for the oxidation of inositol isomers by *Acetobacter suboxydans* can be described in the statement that *only those hydroxyl groups are oxidized that are situated in a polar plane*.

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# STUDIES OF ARTERIOVENOUS DIFFERENCES IN BLOOD SUGAR\*

## I. EFFECT OF ALIMENTARY HYPERGLYCEMIA ON THE RATE OF EXTRAHEPATIC GLUCOSE ASSIMILATION

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The difference between the concentration of glucose in arterial and venous blood (A-V, arteriovenous difference) is largely a reflection of the rate of glucose assimilation in the extrahepatic tissues, mainly in the muscles; any change in the A-V should, therefore, supply valuable information regarding the effects of various factors which influence glucose assimilation. Yet the yield of investigations in this field is rather meager and often confusing. As a matter of fact, the only well demonstrated facts are first, that in lack of insulin (in the depancreatized animal) the A-V is extremely small and, second, that injection of insulin produces an increase in the A-V. Another known fact, namely the increase of A-V in the normal organism during alimentary hyperglycemia, is attributed to an increased insulin supply from the islands of Langerhans.

These facts imply that the magnitude of the A-V is more or less proportional to the insulin supply. According to the current concept of human diabetes, then, the A-V should be invariably lower in diabetic than in normal persons. Clinical studies, however, show that after glucose feeding most diabetics exhibit A-V values as great as, and often greater than, the average normal person does. Unexpected and challenging as these findings are, they failed to elicit any satisfactory explanation.

Even more puzzling are repeated reports of the occurrence of negative A-V values in the postabsorptive state, as well as during alimentary hyperglycemia, in healthy and in diabetic persons. Acceptance of such findings is tantamount to the assumption that muscle cells are capable of secreting glucose into the blood stream. This disregards the well established fact that muscle cells cannot yield free glucose for the simple reason that they do not contain any. This is so, first, because muscle cells are impermeable to free glucose that is transported in the extracellular fluids, and, second, because they possess no enzyme system capable of liberating glucose from the intracellular carbohydrates (glycogen, phosphate esters). Thus negative A-V values can originate but from one of two sources: Either there are certain conditions under which muscle cells secrete into the blood some

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reducing matter other than glucose, or else the analytical techniques employed are unsatisfactory.

The potential value of information that might be derived from accurate A-V determinations prompted a series of studies in our laboratory. But before embarking on this work, we subjected the analytical procedure involved to close scrutiny until, after eliminating several sources of error our technique became accurate within 1 mg. of glucose per 100 cc. of blood. By this method we found only positive A-V values without a single exception. We found, furthermore, certain revealing patterns in the changes of the A-V in response to the influence of hyperglycemia, of insulin action, and of other factors. These changes elude observation without the maximum accuracy of analytical technique that is now attainable. In the present paper we describe the effect of alimentary hyperglycemia on A-V in healthy persons. But before we present our findings, the analytical procedure involved will be discussed in some detail. This we deem necessary, for, without appreciation of the decisive importance of these details, the contradictions between our results and those of many earlier workers could not be properly evaluated.

#### *Analytical Procedure*

For glucose determinations we used the copper-iodometric technique described in 1945 (1). Zinc sulfate and barium hydroxide served for the deproteinization of blood in 1:10 dilution. The venous blood was collected in test-tubes containing uniform mixtures of potassium oxalate and sodium fluoride as anticoagulant. A solution containing 5 per cent of potassium oxalate and 2.5 per cent of sodium fluoride was kept on hand and 0.4 cc. portions of it were pipetted into test-tubes. The water was evaporated and the residue dried at 100–105°. The fluoride was included for the inhibition of glycolysis. This precaution may seem superfluous, considering the fact that the normal rate of glycolysis amounts to only 10 to 14 mg. per cent per hour in oxalated blood, and only a few minutes elapse before the freshly drawn blood is deproteinized. We found, however, that even a few minutes suffice for a measurable decrease of the glucose content of oxalated blood. The rate of glycolysis under the given conditions is far from uniform, a high initial rate occurring in many instances. The phenomenon is rather unpredictable, as there are cases in which glycolysis is not detectable after 5 and even 10 minutes, whereas in others it may amount to as much as 5 mg. per cent in a few minutes. Fluoride obviates this source of error.

The arterial glycemic level was determined in capillary blood. This substitution was found to be satisfactory by Foster (2) and several other investigators. We used 1 cc. portions of capillary blood for each sugar determination, deproteinized in 1:10 dilution, as in the analysis of venous blood. The blood was drawn from a finger-tip and collected in 50 cc.

beakers containing the anticoagulant used for venous blood. (About 0.2 cc. of oxalate-fluoride solution was spread over the bottom of the beakers and the water was expelled by drying at 100–105°.) Collection of finger blood, enough for two glucose determinations in 1 cc. portions of blood, presents no difficulty if the hand is warmed by immersion in water of 45–50° for about 5 minutes before a finger is pricked with a Hagedorn needle. That exposure of the hand to cold or warm baths does not affect the blood sugar was ascertained in our experiments as well as in studies of other workers (3). In the iodometric titrations the discrepancies in duplicate determinations seldom exceeded 0.02 cc. of 0.005 N thiosulfate, which in our method corresponds to 0.54 mg. per cent of blood sugar; rarely did titration differences between duplicates reach 0.04 or 0.05 cc., which correspond to discrepancies of 1.0 to 1.4 mg. per cent in blood sugar values. Thus the differences between the mean and the individual values did not exceed 0.5 to 0.7 mg. per cent. This fact is emphasized as the basis of our statement that the blood sugar values in our experiments are accurate within 1 mg. per cent. Since the A-V determinations involve two sugar determinations, they are susceptible to two analytical errors. While these errors may cancel each other, they also may be additive. In the latter case the cumulative error in the A-V value may amount to as much as 1.0 to 1.5 mg. per cent. We feel justified, therefore, in accepting, rather conservatively, changes in A-V which are greater than 2 mg. per cent to represent actual changes.

It is obvious that such a degree of accuracy is not attainable with micro-methods in which filtrates corresponding to less than 0.5 cc. of blood are used. Provided that the percentage error in the measurement of 0.1 cc. of blood is not greater than in the measurement of 1.0 cc. of blood, the dilution factor alone suffices to limit the accuracy of the results of micromethods. In our method, for example, the dilution factor increases possible errors to 5-fold of the figures stated above if the filtrate of 0.1 cc. (instead of 0.5 cc.) of blood is analyzed. In consequence, when a micromethod is employed and changes in A-V values are not greater than 8 to 10 mg. per cent, they cannot serve as a basis for valid conclusions.

The error introduced by the dilution factor is inseparable from any micromethod, even in the hands of competent analysts. In the Hagedorn-Jensen method, for instance, a titration difference of 0.02 cc. corresponds to 3.6 mg. per cent of blood sugar. But this is true only if the method is used with appropriate modifications, for in its original form, which was widely employed in A-V studies, it opens the door to gross and variable errors, as was demonstrated by Folin and Malmros (4) and by Steiner and Kramer (5). In a word, micromethods cannot be used for studies concerned with A-V, even if the methods are adequate and are employed by competent analysts.

A few additional remarks may be in place concerning the substitution of

capillary for arterial blood sugar. As stated before, we accepted this substitution on the basis of the unanimous opinion of previous workers. Yet for our own satisfaction it was desirable to examine again the older findings with the analytical technique just described. To this end we compared the glucose content of blood samples simultaneously drawn from the femoral artery and the finger-tip of a few hospitalized persons, performing duplicate analyses on 1 cc. portions of both arterial and capillary blood. Each subject was fed 50 gm. of glucose before bleeding in order to produce substantial differences between the arterial and venous glycemic levels. This was done to facilitate the detection of any possible deviation of the capillary blood sugar from the arterial towards the venous blood.

We carried out such analyses only on three persons, because the agreement between the glycemic level in peripheral arterial and in capillary

TABLE I

*Showing Identity of Glucose Concentrations in Peripheral Arterial and Capillary Blood*

Subject	Femoral artery blood	Finger-tip blood	Time after ingestion of 50 mg. glucose
	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>min.</i>
R. B., 38 yrs., male	Lost 359.1	359.4 359.6	35
T. H., 56 " "	135.0 } 134.7 } 134.8	135.0 135.0	
W. C., 18 " "	92.3 } 91.8 } 92.0	92.6 91.8	55

blood was so strikingly complete that it appeared superfluous to expand these experiments further. The identity of the glucose concentration in the femoral arterial blood and in the capillary blood is well demonstrated by the analytical data in Table I. (These figures also illustrate the degree of accuracy attainable in glucose determinations.) It may be noted that discrepancies in all three cases are less than 1 mg. per cent. Yet, in the other experiments reported in this paper we have not recorded fractions of a mg.; the glucose values, we felt, present the facts adequately enough with the decimal fractions rounded out to the nearest whole number.

Although we invariably analyzed capillary instead of arterial blood, we have denoted the capillary glucose values as arterial blood sugar and speak of arteriovenous rather than of capillary-venous differences. This seeming inaccuracy of terminology should be tolerated for the following reasons: In the first place, this has been the general usage in all publications, including articles in this *Journal*, during the past 25 years, and we did not feel

justified in introducing a departure from this usage without compelling reasons. Secondly, there is no reason for the departure, since the actual identity of arterial and capillary blood sugar values precludes any misunderstanding or confusion.

### *A-V Difference in Postabsorptive State*

As a basis for further observations it is well to record here the A-V we found in healthy individuals in the postabsorptive state (10 to 14 hours after the last meal). In Table II are presented our last 100 individual cases, grouped according to increasing values. It may be noted that the A-V is positive in every instance; *i.e.*, the arterial blood sugar is always higher than the venous. This is in line with the well recognized fact that muscle

TABLE II

*Arteriovenous Differences in 100 Healthy Individuals in Postabsorptive (Fasting) State*  
The values represent mg. per 100 cc. of blood.

No. of cases	A-V	Postabsorptive glucose in arterial blood
9	1	82-96
11	2	79-97
12	3	84-92
21	4	78-93
17	5	79-96
13	6	87-95
10	7	86-96
1	8	90
3	9	85-92
3	10-13	88-94

cells continually assimilate blood sugar, and hence the concentration of glucose inevitably decreases during the passage of blood through the tissues. But the A-V values are rather small, only from 1 to 7 mg. per cent in 93 of 100 cases. Thus negative A-V values can easily result from relatively slight analytical errors (see the preceding section).

The magnitude of the A-V is not an individual characteristic; it may vary within the normal limits from day to day in any healthy person. Nor is it related, as it may be noted in Table II, to the postabsorptive glycemic level.

### *Effect of Alimentary Hyperglycemia*

Hyperglycemia produced either by oral or by parenteral administration of glucose always entails a substantial increase in the A-V, a manifestation of an increased rate of glucose assimilation in the muscle tissues. This change is generally attributed to increased insulin action, due to the aug-

mented secretory activity of the islands of Langerhans under the stimulating effect of hyperglycemia. The A-V values are without exception positive. The large negative values often encountered in the literature (6-8) are undoubtedly the results of analytical errors.

In Table III are presented some of the results accumulated in our laboratory during the last 2 years. They are examples of observations on forty-four healthy individuals, in whom the changes in the sugar level in both the arterial and venous blood were followed for a period of 4 hours after the

TABLE III

*Effect of Alimentary Hyperglycemia on Arteriovenous Difference*

The values represent mg. per 100 cc. of blood.

Serial No. of subject	Sex, age	Arterial blood sugar, after ingestion of glucose						A-V, after ingestion of glucose					
		0 hr.	0.5 hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	0 hr.	0.5 hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.
21	F., 25 yrs.	94	133	118	103	99	99	7	37	35	28	23	23
23	" 23 "	96	146	95	118	108	99	7	37	24	29	34	17
3	" 24 "	88	148	115	108	94	68	4	40	25	28	13	5
22	" 22 "	92	149	122	116	122	119	4	43	23	31	21	25
32	M., 33 "	88	134	149	116	106	77	1	20	20	18	13	2
2	" 27 "	83	154	148	107	80	73	2	28	49	33	16	3
27	F., 30 "	97	155	138	151	126	73	2	35	33	29	35	6
17	M., 41 "	88	163	142	122	118	73	4	22	23	29	16	2
19	" 29 "	84	169	153	165	142	80	3	22	30	23	20	6
18	F., 40 "	90	170	126	120	80	92	1	20	18	20	10	12
38	M., 32 "	91	170	142	123	64	70	9	52	48	31	3	1
15	" 38 "	94	173	169	100	68	79	6	23	48	30	18	2
7	F., 38 "	85	160	176	146	128	115	3	37	55	34	25	22
16	M., 49 "	94	181	143	105	83	57	11	48	33	11	6	4
25	" 28 "	88	185	141	111	72	69	13	53	38	12	9	7
34	F., 40 "	102	176	190	145	144	62	4	27	50	33	13	1

oral administration of 100 gm. of glucose under standardized conditions. Cases in which the hyperglycemic peak rose above 150 mg. per cent in the venous, or above 190 mg. per cent in the arterial blood, were excluded from the normal series, since these higher peaks were regarded as signs of abnormally diminished glucose tolerance. We also ruled out those in which the blood sugar continued to rise during the 2nd hour after the ingestion of glucose.

To save space, the values for venous blood sugar were not included in Table III, since they can be readily obtained by deducting the A-V values from those for the arterial blood sugar. For the same reason, only about one-third of the cases is presented; this gives a satisfactory picture of our

findings. The experiments are arranged according to increasing peaks in the hyperglycemia for reasons which will be clear presently.

A glance at Table III discloses great individual variations, a fact that is by no means surprising. The only pronounced uniformity, with only occasional discrepancies, consists of the prompt and steep increase in the A-V as soon as the blood sugar begins to rise, and a somewhat less consistent decline of the A-V as the hyperglycemia subsides. As a rule, a maximum A-V value is attained within 0.5 to 1 hour after the ingestion of the glucose.

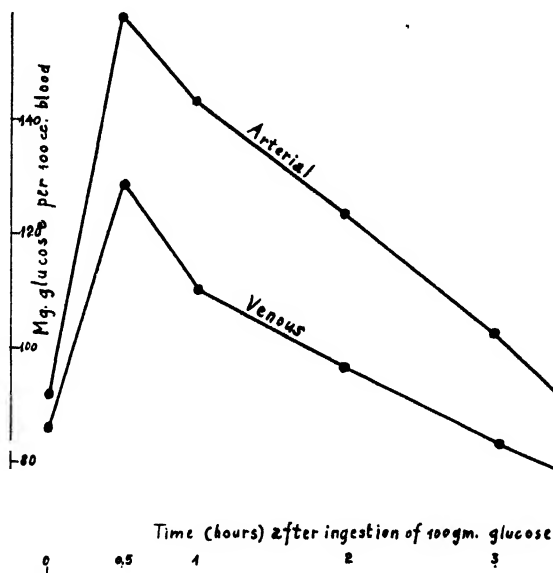


FIG. 1. Composite glucose-time curves (tolerance curves) in arterial and venous blood of forty-four healthy persons during 4 hours after the ingestion of 100 gm. of glucose.

But from this point on individual variations in the relation between the glycemic level and the A-V are evident, with one notable exception. That is, the A-V remains consistently higher than its initial (postabsorptive) value, and returns to the latter, or below it, only after the glycemic level has declined below its postabsorptive level.

A general pattern of the responses to glucose feeding is presented in Figs. 1 and 2, which represent the average values of the forty-four experiments of this series. In Fig. 1 it may be noted that statistically the hyperglycemic peak occurs 0.5 hour after the ingestion of glucose, both in the arterial and in the venous blood, and that the peak is followed by a continuous decline until the glycemic level drops below the postabsorptive level at the end of



the 4th hour. The average A-V (Fig. 2) follows a similar pattern, but its changes show a discernible lag compared with the changes of the blood sugar curves. This lag is manifest in a slight but discernible increase in the A-V during the second half hour period, when the blood sugar is already declining. A similar lag is noticeable in the declining phase of the curves; at the 4 hour period, when the blood sugar curves dip below the fasting level, the A-V is still slightly greater than the initial value. This chronologic lag is but an expression of the fact that the changes in A-V are the functions of the changes in the glycemic level.

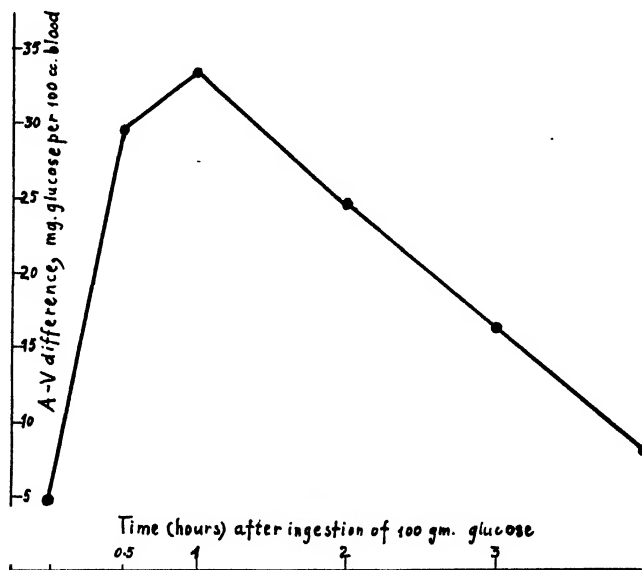


FIG. 2. Composite curve representing the changes in arteriovenous difference in forty-four healthy persons during 4 hours after the ingestion of 100 gm. of glucose.

That alimentary hyperglycemia entails increased A-V values is a well recognized fact. Our experiments now show that this quantitative relationship is more intimate than it was considered previously. We found that the higher the peaks of the hyperglycemia, the greater is the maximum A-V. This relationship is discernible in Table III, but, owing to the natural constitutional differences between the subjects in these experiments, this relationship is not readily apparent. On closer scrutiny, however, it is noticeable that the highest A-V tends to occur in those cases in which the hyperglycemic peaks are highest (bottom group of Table III). That this is the general rule became clearer when we classified our forty-four cases into three groups: the first with hyperglycemic peaks ranging between 131 and 150 mg. per cent, the second with peaks between 151 and 170, and the

third with peaks between 171 and 190 mg. per cent. The respective averages of the hyperglycemic peaks in the three groups then were correlated with the averages of the corresponding maximum A-V, as presented in Table IV. It may be seen that in the first two groups, in which the average hyperglycemic peaks are 141 and 163 mg. per cent, respectively, the maxi-

TABLE IV

*Quantitative Relationship between Height of Hyperglycemic Peak in Arterial Blood and Magnitude of Arteriovenous Difference*

The values represent mg. per 100 cc. of blood.

No. of cases	Peak of hyperglycemia in arterial blood		A-V maximum	
	Range	Average	Range	Average
12	131-150	141	20-50	34
24	151-170	163	19-52	35
8	171-190	182	31-58	48

TABLE V

*Higher Hyperglycemic Peaks Entail Higher Arteriovenous Differences in Same Individual*

The numbers in bold-faced type indicate peaks.

Glucose ingested	Time after ingestion of glucose	Glucose		A-V
		Arterial blood	Venous blood	
gm.	min.	mg. per 100 cc.	mg. per 100 cc.	mg. per cent
10	0	84	79	5
	10	101	93	8
	20	<b>120</b>	109	<b>11</b>
	30	121	111	10
	45	109	98	11
25	0	82	79	3
	10	113	103	10
	20	146	127	19
	30	<b>159</b>	129	<b>30</b>
	45	153	126	27

mum A-V values are much the same, in seeming contradiction to our contention. In the third group, however, with an average peak of 182 mg. per cent, the maximum A-V steeply rises from 35 mg. per cent to 48 mg. per cent.

In this survey the material was not sufficiently extensive to suppress the influence of individual variations. We eliminated this factor by producing different degrees of alimentary hyperglycemia in one and the same indi-

vidual. In one group of subjects this was attained by the oral feeding of variable amounts of glucose. Two examples of such experiments are presented in Tables V and VI. Table V shows that after ingestion of 10 gm. of glucose, when the hyperglycemic peak was 120 mg. per cent, the maximum A-V was 11, whereas, when the ingestion of 25 gm. of glucose increased the hyperglycemic peak to 159 mg. per cent, the maximum difference was almost trebled; i.e., it rose to 30 mg. per cent. The other example, given in Table VI, shows a similar quantitative relationship at three different hyperglycemic levels. The slight hyperglycemia of 111 mg. per cent, elicited by the ingestion of 5 gm. of glucose, produced the barely discernible increase

TABLE VI

*Consistent Increase of Maximum Arteriovenous Difference with Increasing Hyperglycemic Peaks in Same Individual*

The numbers in bold-faced type indicate peaks.

Glucose ingested	Time after ingestion of glucose	Glucose		A-V
		Arterial blood	Venous blood	
gm.	min.	mg. per 100 cc.	mg. per 100 cc.	mg. per cent
5	0	91	91	1
	15	<b>111</b>	110	1
	30	102	100	2
	45	94	90	<b>4</b>
25	0	88	84	4
	15	122	110	12
	30	<b>171</b>	155	16
	45	168	151	<b>17</b>
100	0	87	85	2
	30	182	150	32
	45	<b>195</b>	157	<b>38</b>

of 3 mg. per cent. After the ingestion of 25 gm. of glucose the hyperglycemic peak was 171, and after 100 gm. of glucose it was 195 mg. per cent; the corresponding maximum A-V values were 17 and 38 mg. per cent, respectively.

Since the hyperglycemic peak cannot be raised beyond a certain maximum level by oral feeding of glucose (the maximum being produced by the ingestion of from 25 to 50 gm. of glucose), we resorted to the intravenous administration of glucose in order to see whether or not abnormally increased hyperglycemia elicits an abnormally high A-V. In one experiment, the results of which are presented in Table VII, a healthy person was fed at one time 100 gm. of glucose by mouth, and on another occasion 50 gm. of glucose were given by mouth and at the same time 100 gm. of glucose

(in 10 per cent solution) were injected intravenously at a constant rate in the course of 2 hours. As may be seen, after oral administration the hyperglycemic peak was 166 mg. per cent, while by additional intravenous administration an abnormally high peak of 286 mg. per cent was produced. The corresponding maximum A-V values were 36 and 85 mg. per cent, respectively. These observations demonstrate unequivocally the fact that in healthy individuals the A-V increases with increasing levels of alimentary hyperglycemia.

TABLE VII  
*Exaggerated Hyperglycemic Levels Entail Exaggerated  
Arteriovenous Differences in Healthy Person*

The numbers in bold-faced type indicate peaks.

Time after administration of glucose	Glucose		A-V
	Arterial blood	Venous blood	
Apr. 19, 1946, after ingestion of 100 gm. glucose			
hrs.	mg. per 100 cc.	mg. per 100 cc.	mg. per cent
0	95	89	6
0.5	136	105	31
1	166	130	36
2	151	134	17
3	145	126	19
May 7, 1946, after ingestion of 50 gm. glucose and simultaneous intravenous infusion of 100 gm. at rate of 50 gm. per hr.			
0	90	88	2
0.5	250	193	67
1	286	201	85
2	277	217	60
3	105	87	18

*Rôle of Extrahepatic Tissues in Homeostasis of Glycemic Level*

This phenomenon reveals the important rôle of the extrahepatic (mainly muscle) tissues in the control mechanism which regulates the blood sugar, with the tendency to balance its concentration as near as possible at the constant postabsorptive level. The part of the liver in the performance of this task has been well known since Claude Bernard. Lately it was demonstrated in well controlled experiments (9) that the higher the hyperglycemic level, the greater is the rate of glucose assimilation in the liver. In other words, increase in the glucose concentration of the blood entails an increase in the amount of glucose which the liver removes from the blood. This is a potent factor in the control of the glycemic level. This fact has occa-

sionally been emphasized in such a manner as to convey the impression that the liver is the only important factor in the homeostasis of the glycemic level (10). The response in the extrahepatic tissues to increasing blood sugar concentrations, as shown in the preceding section, is the same as in the liver. This makes it understandable that during alimentary hyperglycemia the muscles remove even more glucose from the blood than the liver does (11), and thus their function in the homeostasis of the glycemic level is by no means less significant than that of the liver.

#### SUMMARY

Determinations of the differences between glucose concentrations in arterial (capillary) and venous blood (A-V difference) of healthy persons in the postabsorptive state and during alimentary hyperglycemia are presented.

The glycemic level is always higher in the arterial than in the venous blood; this holds true in the postabsorptive state as well as during alimentary hyperglycemia. Reversal of this relationship was not encountered; *i.e.*, the difference, arterial blood sugar minus venous blood sugar, is always a positive quantity. The average value in the postabsorptive state is 4.8 mg. per cent. The average of the maximum A-V, statistically occurring 1 hour after feeding 100 gm. of glucose, was 33.5 mg. per cent; the highest value was 58 mg. per cent.

The increase of the A-V is a function of the hyperglycemic level; *i.e.*, the higher the hyperglycemic peak, the greater becomes the A-V.

The importance of the adequacy of the analytical technique in studies of A-V is emphasized.

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# THE DETERMINATION OF DIKETO-L-GULONIC ACID, DEHYDRO-L-ASCORBIC ACID, AND L-ASCORBIC ACID IN THE SAME TISSUE EXTRACT BY THE 2,4-DINITROPHENYLHYDRAZINE METHOD\*

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By the Roe and Kuether (1) method for the determination of vitamin C dehydro-l-ascorbic (DHA) and diketo-l-gulonic acid (DKA) are not differentiated. The basic principle of this method, however, is adaptable to the determination of l-ascorbic acid (AsA), DHA, and DKA in the presence of each other. It is the purpose of this paper to report methods for such determinations.

The first problem to overcome in this work was the extraction of the three compounds from tissues. This must be done by techniques that will not permit the oxidation of AsA to DHA and will keep at a minimum the change of DHA into DKA. Roe and Oesterling (2) prevented the oxidation of AsA in plant tissues by extracting with a metaphosphoric acid solution containing 1 per cent of thiourea. However, in animal tissues thiourea will not adequately prevent the oxidation of AsA during extraction because of the powerful oxidant effect of oxyhemoglobin. The problem was solved by adding  $\text{SnCl}_2$  in 10 per cent concentration to the  $\text{HPO}_3$  solution;  $\text{SnCl}_2$  effectively reduces the oxyhemoglobin of animal tissues. Furthermore  $\text{SnCl}_2$  is a more convenient reagent, since it can be removed later in the procedure.

The procedure developed consists of grinding the tissue under 10 per cent solution of stannous chloride in 5 per cent  $\text{HPO}_3$ , after which enough 5 per cent metaphosphoric acid solution is added to make the  $\text{SnCl}_2$  concentration 0.5 per cent.

From data on melting points, nitrogen content, and chromatographic adsorption Penney and Zilva (3) came to the conclusion that the 2,4-dinitrophenylhydrazine derivatives of AsA, DHA, and DKA are the same compound. Absorption curves of the derivatives of DHA and DKA, prepared by ourselves, also indicate that these derivatives are identical substances. Penney and Zilva (3) have postulated that 2,4-dinitrophenylhydrazine couples only with DKA and, therefore, DHA gives rise to a derivative in

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the presence of this reagent only after conversion to DKA. Our studies support this hypothesis of Penney and Zilva.

To determine DKA in the presence of AsA and DHA through the 2,4-dinitrophenylhydrazine derivative one might make use of the more rapid rate of coupling of DKA with 2,4-dinitrophenylhydrazine, as Penney and Zilva did (3), or one might reduce the DHA with  $\text{H}_2\text{S}$  and then couple the unchanged DKA with 2,4-dinitrophenylhydrazine. The  $\text{H}_2\text{S}$  procedure is the classical method for differentiating between the two compounds and for analytical work it was found by us to be more sensitive, adaptable, and reliable.

✓ Analysis of metaphosphoric acid-stannous chloride filtrates of tissues containing AsA, DHA, and DKA by the 2,4-dinitrophenylhydrazine procedure gives a composite value which includes DHA and DKA (called Determination 2). Appropriate treatment of this filtrate with  $\text{H}_2\text{S}$  reduces the DHA and leaves the DKA almost completely unchanged. Analysis of this filtrate by the dinitrophenylhydrazine method gives the DKA content (Determination 1). Subtracting this result from the value of Determination 2 gives the DHA. Bromine oxidation of the  $\text{H}_2\text{S}$  filtrate, after removal of  $\text{H}_2\text{S}$ , yields a solution containing all of the vitamin C in the oxidized forms. Analysis of this solution by the dinitrophenylhydrazine method gives a composite value including AsA, DHA, and DKA (Determination 3). Subtracting the value for Determination 2 from that for Determination 3 gives the AsA content of the original extract.

✓ *Factors Affecting Coupling Rate*—Since initially DKA solution produces more derivative with 2,4-dinitrophenylhydrazine than does DHA solution, it was necessary to set up conditions that will permit the coupling to go practically to completion. We use a strongly acid reaction mixture to promote coupling, as the rate of change of DHA into DKA is accelerated by the hydrogen ion. In our reaction mixture the coupling of DHA with 2,4-dinitrophenylhydrazine in 1 hour at  $37^\circ$  is 70 per cent of that of a molar equivalent of DKA with the same reagent. As is indicated in Fig. 1, the curves showing coupling of DHA and DKA are very close at the 5 hour period and practically superimposed upon each other at 14 hours.

However, AsA will show slight coupling at  $37^\circ$  in a prolonged incubation period. Since error begins to occur from coupling with ascorbic acid after 8 hours of incubation, a 6 hour incubation period was adopted as most satisfactory for our procedure. The coupling of DHA is 95 per cent of that of DKA during this time, and this error is constant and can be corrected for in accurate work.

The rate of coupling is influenced by the concentration of the reducing agent present in the reaction mixture (4). Since in Determination 1 the  $\text{SnCl}_2$  is removed by  $\text{H}_2\text{S}$  treatment, it is necessary to add another reducing

agent to prevent reoxidation of AsA and to provide the correct medium for coupling with 2,4-dinitrophenylhydrazine. For this purpose we use thiourea in a concentration of 1 per cent, which was found to yield essentially the same coupling curve as 0.5 per cent  $\text{SnCl}_2$ .

*Conditions for Complete Reduction of Dehydroascorbic Acid*—Most workers have utilized a pH of 3.5 when reducing DHA with  $\text{H}_2\text{S}$ . This is an optimum condition if DHA is the only oxidation product present. In an attempt to establish what conditions blocked the reduction of DHA in the presence of  $\text{H}_2\text{S}$  and  $\text{SnCl}_2$  we worked at various acidities from pH 0.4 to 7.0. pH readings were taken after removal of the  $\text{SnS}$  by filtration. The curves given in Fig. 2 show that reduction with  $\text{H}_2\text{S}$  for 15 minutes results

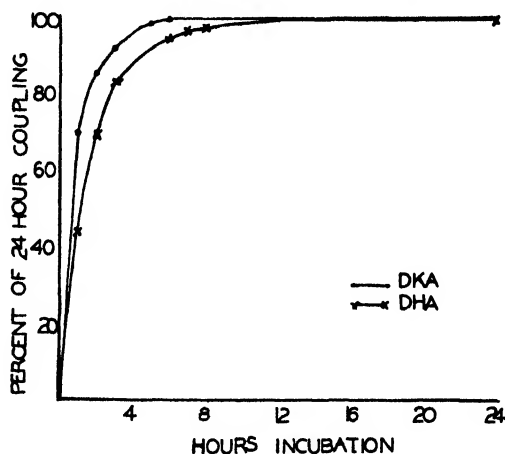


FIG. 1. Coupling rates of DHA and DKA. Standardized DHA and DKA solutions, containing 1 per cent thiourea, were incubated with 2,4-dinitrophenylhydrazine at  $37^\circ$ .

in complete regeneration of AsA at pH ranges from 4.7 to 1.2. Below pH 1.2, the reduction is sharply diminished. Reduction of DHA was practically complete in 5 minutes at pH 3.5, but required 15 minutes at pH 1.2 to 2.0. It is suggested that the slower reduction of DHA at low pH values is due to a decreasing  $\text{H}_2\text{S}$  concentration with increasing acidity, although at very low pH values the failure may be due in part to a conversion of DHA to DKA (Fig. 3). Other reducing reagents were tried, including  $\text{SnCl}_2$  and  $\text{Na}_2\text{S}$ , but none proved as satisfactory as  $\text{H}_2\text{S}$ .

✓ *Conditions for Protection of DKA*—In the presence of  $\text{H}_2\text{S}$ , DKA is slowly converted into an unknown product (5). This change is not a reduction to AsA, since subsequent reoxidation does not restore the original value.

In studying this conversion we first determined the amount of DKA



remaining in a solution after exposure to  $\text{H}_2\text{S}$  for different lengths of time, at several pH values, as shown in Fig. 4. It can be seen that DKA is slowly

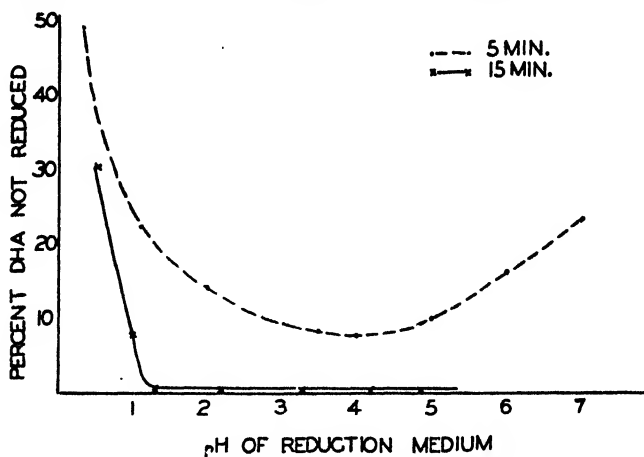


FIG. 2. Reduction of DHA at various pH values. Fresh DHA solution was pipetted into buffers of 5 per cent  $\text{HPO}_3$  adjusted with concentrated  $\text{HCl}$  or solid  $\text{Na}_2\text{CO}_3$ . The solutions were reduced immediately with  $\text{H}_2\text{S}$  for 5 or 15 minutes; pH values were taken after reduction.

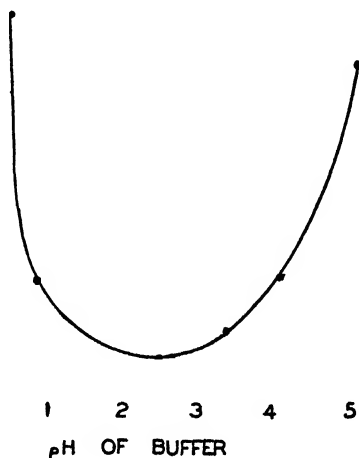


FIG. 3. Conversion of DHA to DKA at various pH values. Fresh DHA solution was pipetted into buffer solutions  $\frac{1}{2}$  hour before analysis. The pH was adjusted to 1.25 before reduction.

diminished as a direct function of time. The change is also seen to occur with greater rapidity at increasing pH values. Protection at pH 1.3 was approximately 97 per cent with 15 minutes exposure to  $\text{H}_2\text{S}$ .

This leaves a fairly restricted pH at which to work for complete reduction of DHA and satisfactory protection of DKA. We found the range of pH 1.25 to 1.30 to be safest, and since this is the pH of fresh 5 per cent  $\text{HPO}_3$ , the latter concentration of acid was adopted for use.

#### *Analytical Procedure*

✓ *Extraction*—Tissue is thoroughly ground in a mortar under 1 volume of 10 per cent solution of  $\text{SnCl}_2$  in 5 per cent  $\text{HPO}_3$ . 19 volumes of 5 per cent  $\text{HPO}_3$  solution are added. (Do not use a Waring blender or homogenizing apparatus that would introduce increased amounts of oxygen into the slurry.) The slurry is thoroughly mixed and filtered. A solution with a

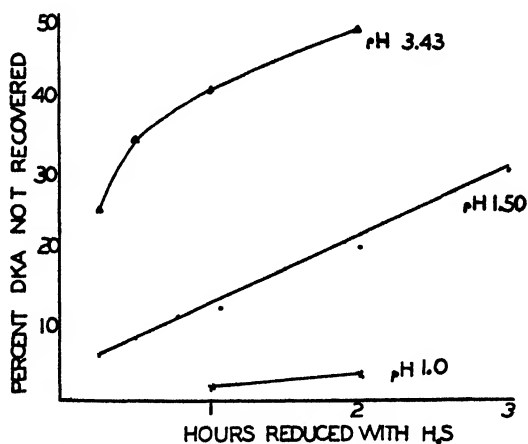


FIG. 4. Per cent DKA not recoverable by the 2,4-dinitrophenylhydrazine method after  $\text{H}_2\text{S}$  treatment. Solutions in 5 per cent  $\text{HPO}_3$  were adjusted to different pH values with solid  $\text{Na}_2\text{CO}_3$  or concentrated  $\text{HCl}$  and exposed to  $\text{H}_2\text{S}$  at  $25^\circ$  for varying periods up to 3 hours.

dilution of 1 part of tissue in 100 parts of extract is desirable and in dilutions greater than this a concentration of 1 to 10  $\gamma$  per cc. of total AsA, DHA, and DKA is recommended. It is necessary to work rapidly to prevent changes in partition of the three compounds. A conversion of DHA to DKA of 5.7 per cent in a solution containing 0.5 per cent  $\text{SnCl}_2$ -5 per cent  $\text{HPO}_3$  was observed in 1 hour at room temperature.

✓ *Determination of DKA*—Place 100 cc. of the original  $\text{SnCl}_2$ - $\text{HPO}_3$  filtrate in a large test-tube. Introduce a gas filter tube with a sintered glass filter about 20 mm. in diameter and pass  $\text{H}_2\text{S}$  through for 15 minutes. To 40 cc. of the  $\text{H}_2\text{S}$ -saturated extract add 0.4 gm. of powdered thiourea, shake until the latter is dissolved, and filter. Into the filtrate bubble  $\text{CO}_2$  for 5 minutes. Pipette 4 cc. aliquots of this solution into each of three photoelectric colori-

meter tubes. Add 1 cc. of 2 per cent 2,4-dinitrophenylhydrazine in 9 N  $\text{H}_2\text{SO}_4$  to two of these tubes and hold one tube as a blank. Place the three tubes in a water bath at  $37^\circ$  for 6 hours. From here on the analysis is the same as that described by Roe and Kuether (1). The value obtained here is the DKA content.

✓ *Composite Determination of DHA and DKA; for DHA Content*—4 cc. aliquots of the original  $\text{SnCl}_2\text{-HPO}_3$  filtrate are pipetted into each of three colorimeter tubes. 1 cc. of 2 per cent 2,4-dinitrophenylhydrazine in 9 N  $\text{H}_2\text{SO}_4$  is pipetted into two of the tubes, the third tube being held as a blank. The three tubes are placed in a water bath at  $37^\circ$  for 6 hours. The analysis from here on is identical with that described by Roe and Kuether (1). From the value obtained here subtract the DKA value determined above to get the DHA content.

✓ *Composite Determination of AsA, DHA, and DKA; for AsA Content*—The remainder of the  $\text{H}_2\text{S}$ -saturated solution is filtered to remove  $\text{SnS}$  (thiourea is not added) into a test-tube suitable for bubbling gas. Air is drawn through this solution until the  $\text{H}_2\text{S}$  is removed. The air should be drawn through a water trap before passing through the  $\text{H}_2\text{S}$  solution to minimize volume changes. Sufficient bromine is added to color the solution. Excess bromine is removed by bubbling air through the solution as above. Powdered thiourea in quantities sufficient to make a 1 per cent solution is added. 4 cc. aliquots of this solution are pipetted into each of three colorimeter tubes. 1 cc. of 2 per cent 2,4-dinitrophenylhydrazine in 9 N  $\text{H}_2\text{SO}_4$  is added to each of two tubes and the third tube is held as a control. The three tubes are placed in a water bath at  $37^\circ$  for 6 hours. From here on proceed as described by Roe and Kuether (1). The result obtained here is a composite value including AsA, DHA, and DKA. From this value subtract the composite value for DHA and DKA obtained above. This gives the AsA content.

✓ *Preparation of Calibration Curves*—Since in the above procedures the rate of coupling is influenced by the acid solution used and the antioxidant present, it is important in preparing a calibration curve to use the same concentration of  $\text{HPO}_3$  and thiourea or  $\text{SnCl}_2$  as is used in the procedure followed for the unknown. We use a calibration curve made with standard dehydroascorbic acid solution prepared by bromine oxidation of ascorbic acid as directed by Roe and Oesterling (2). The standards are made up in 5 per cent  $\text{HPO}_3$  solution containing 1 per cent of thiourea. The tubes are incubated for 6 hours at  $37^\circ$ . The curve obtained with 1 per cent thiourea is practically the same as that obtained with 0.5 per cent  $\text{SnCl}_2$ . Therefore, calculations of DHA, DKA, and AsA are all made with the same standard curve.

*Recovery Studies*

The method outlined above was tested on solutions of known concentration. Recoveries are shown in Table I. The AsA solution was prepared by dissolving crystalline ascorbic acid<sup>1</sup> in 5 per cent  $\text{HPO}_3$ . The DHA solution was prepared by dissolving the crystalline ascorbic acid in distilled water, oxidizing with bromine, and removing the excess oxidant by bubbling air

TABLE I  
*Recovery of Ascorbic, Dehydroascorbic, and Diketogulonic Acids from Solutions of Known Concentrations*

		AsA	DHA	DKA
		$\gamma$ per 4 cc	$\gamma$ per 4 cc.	$\gamma$ per 4 cc.
Pure solutions	Amount present	36.7	0.0	0.0
	Found by method	36.4	0.2	0.0
	Amount present	0.0	35.5	0.0
	Found by method	0.0	35.2	0.4
	Amount present	0.0	0.0	61.2
	Found by method	0.0	0.2	60.6
	Amount present	14.5	0.0	19.4
	Found by method	14.0	0.2	19.3
Additions to blood	Amount present	0.0	18.7	21.0
	Found by method	0.0	18.8	20.7
	Amount present	35.2	0.0	0.0
	Found by method	35.1	0.1	0.0
	Amount present	0.0	29.5	0.0
	Found by method	0.0	27.6	0.5
	Amount present	0.0	0.7	24.2
	Found by method	0.0	0.6	24.5
Additions to spinach	Amount present	33.4	1.4	0.0
	Found by method	33.5	1.0	0.2
Homogenization with Waring blender	Amount present	40.0	0.0	0.0
	Found by method	29.0	10.0	0.9

through the solution for 5 minutes. The DKA solution was made from calcium diketogulonate prepared by a modification of the Penney and Zilva method (6). Additions of AsA, DHA, and DKA were made to blood because the latter presents the most critical test of the method owing to its high oxyhemoglobin content. A loss of about 4 per cent of dehydroascorbic acid added to blood is shown in Table I, which seems unavoidable even with the use of such protective reagents as  $\text{SnCl}_2$ ,  $\text{H}_2\text{S}$ , and thiourea. The recoveries obtained with blood, however, are good in view of the fact that the

<sup>1</sup> Kindly supplied to us by Merck and Company, Inc.

amounts added were 100 times in excess of the amount normally present. Recoveries of the three forms of the vitamin added to other animal tissues were entirely satisfactory.

This method has been successfully applied to analyses of ascorbic acid and its oxidation products in fresh plant and animal tissues and in processed foods. The results will be reported later.

#### SUMMARY

A method is presented for the determination of diketo-*l*-gulonic acid, dehydro-*l*-ascorbic acid, and *l*-ascorbic acid, each in the presence of the others, by means of the 2,4-dinitrophenylhydrazine method.

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## DISTRIBUTION STUDIES

### X. ATTAINMENT OF EQUILIBRIUM

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One of the requisites for the application of the counter-current distribution procedure to any solute or mixture of solutes is the establishment of complete equilibrium between the two phases at each step. This is necessary if the partition ratio is to be used for the calculation of a theoretical curve (1) and in most cases is advisable also in order to achieve the best separation possible with a mixture of solutes. Thus far in our work equilibrium has been established by inversion of the barrel of the distribution apparatus, then righting it again, 50 times before the phases were allowed to separate. The rate of inversion was so adjusted that good dispersion was maintained. That essential equilibrium had been established was made certain by the good agreement between experimental and calculated curves (1). With the possible exception of one instance, no case has yet been found where 50 inversions have not given essential equilibrium.

However, the possibilities inherent in the use of higher numbers of transfers are being explored and for this purpose any reduction in the time required at each step becomes of increased importance. Moreover, with unstable solutes such as the penicillins the time of the distribution should be reduced as much as possible in order to minimize the percentage of transformation. A simple quantitative method of studying the rate at which equilibrium is established has therefore been devised which simulates the equilibration conditions of the distribution apparatus as nearly as possible.

In this procedure the equilibration vessel was a glass-stoppered Pyrex tube with approximately the inside dimensions of one of the tubes of the distribution apparatus; *i.e.*, 7.5 inches in length and 0.5 inches in diameter. An arbitrary weight of the solute was dissolved in one of the phases and placed in the tube. The other phase was carefully stratified above or below the one previously inserted in the tube so as to avoid mixing. The tube was then attached to the barrel of the distribution apparatus and an arbitrary number of standard inversions was applied. The two phases usually separated in about 30 seconds. The amount of solute in each phase was then determined by some suitable analytical method and the ratio of the

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concentrations was recorded. The procedure was repeated successively with varying numbers of inversions. The logarithms of the ratios so obtained were plotted as ordinates against the number of standard inversions as abscissa as in Fig. 1. A second curve was then determined in the same manner, except that the solute in this case was initially dissolved in the other phase.

An example of such a study is given with benzylpenicillin in the system ethyl ether-3 *m* phosphate buffer at pH 4.60 (System 2 (2)). For each

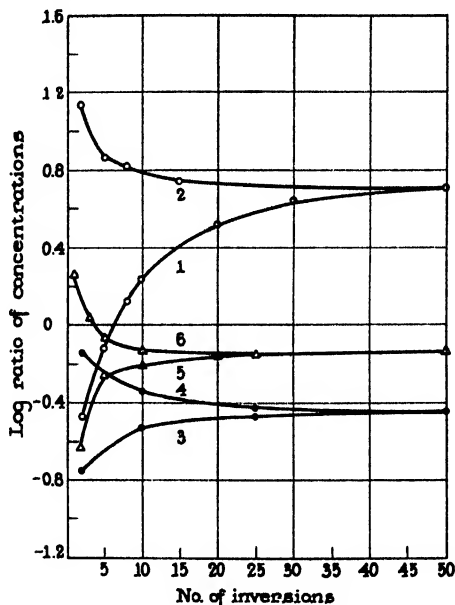


FIG. 1. Rate of establishment of equilibrium. Curves 1 and 2; benzylpenicillin in ethyl ether-3 *m* phosphate buffer at pH 4.60; Curves 3 and 4, *p*-hydroxybenzylpenicillin in ethyl ether-3 *m* phosphate buffer at pH 4.9; Curves 5 and 6, benzylpenicillin in ethyl ether-2 *m* phosphate buffer at pH 4.86.

determined point of Fig. 1 approximately 10 mg. of the sodium salt were used. The concentrations were derived spectroscopically at a wave-length of 260  $m\mu$  with the Beckmann quartz spectrophotometer. The equilibrations were made in the cold room at 5–6° with 8 cc. of each of the phases. Obviously the point at which Curves 1 and 2 merge will give the number of inversions required for essential equilibrium.

Although the data given in Fig. 1 were sufficient to permit selection of the optimum operating conditions with the distribution apparatus for the particular case, it appeared that the investigation in itself was deserving of further study. It was soon found that the values obtained could be repro-

duced readily in different tubes with similar dimensions. The amount of solute taken initially within certain limits was not critical. It proved unnecessary to attach the glass tube to the distribution apparatus in order to reproduce the result. Beyond these conditions almost any other change had its effect.

The number of inversions required for the pair of curves to merge was found to depend strongly on the relative volumes of the phases, on the value of the partition ratio, on the temperature, on the particular solute involved, and on the type of system used. Thus, the result obtained with *p*-hydroxy-

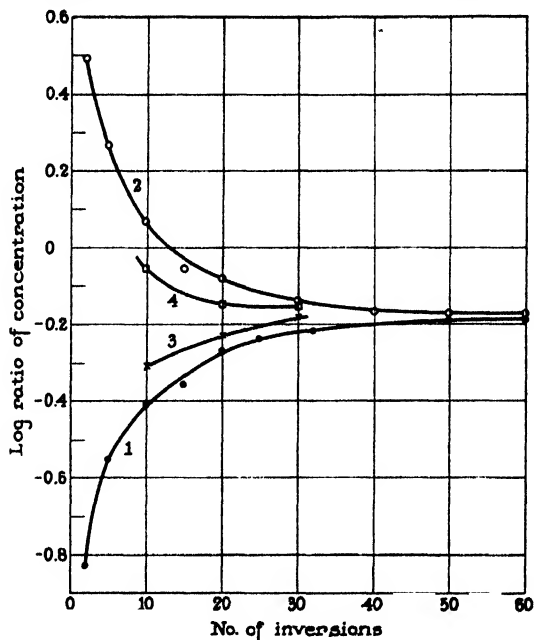


FIG. 2. Rate of establishment of equilibrium. Benzylpenicillin in isopropyl ether-3 M phosphate buffer at pH 4.9. Curves 1 and 2 at 5-6°, Curves 3 and 4 at 24°.

benzylpenicillin in ethyl ether-3 M phosphate at pH 4.9 (System 3 (2)) is shown in Curves 3 and 4 of Fig. 1 and that with benzylpenicillin in ethyl ether-2 M phosphate at pH 4.86 (System 6 (2)) is given by Curves 5 and 6.

In Fig. 2 benzylpenicillin has been studied in isopropyl ether-3 M phosphate at pH 4.9, 13 and 7 cc. respectively. The inversions were made at 5-6° for Curves 1 and 2 and at 24° for Curves 3 and 4. As might be expected, increasing the temperature increased the rate at which equilibration was established.

The problem of the rate of interchange of a solute between two immiscible liquids in intimate contact is one of considerable interest, particularly for



the industrial chemist and physiologist. Accordingly, a considerable literature exists on this subject (3, 4) in which definite resistance to the immediate establishment of equilibrium has quite generally been noted. The experimental observations appear to be interpreted best in terms of the "two film" theory. A discussion of this rather involved theory would appear scarcely warranted in the present connection. However, the results we have obtained all would appear to be in line with such a theory.

As would be expected from the theory, gentle stirring of the two layers in the tube by slow tilting or inversion, so that the phases were not dispersed into tiny droplets, has given a very poor rate of interchange. On the other hand, a quick inversion followed by a slight pause before the next inversion would appear to offer the optimum conditions for interchange, since the droplets begin to coalesce during the pause only to be broken up again at the next inversion. This could, on the basis of the "two film" theory, well reduce the resistance to interchange of the solute at an interface to a minimum.

In connection with such a view-point, it might be interesting to speculate on the effect an emulsifying agent might have on the rate of interchange of solute, since here, although the droplet is very small, its surface could be stabilized, have greater viscosity, and would be more like a solid. It is conceivable that such a condition might not give good interchange of solute. Although we do not have quantitative data on this point, we have frequently noted that an experimentally determined partition ratio was difficult to duplicate when the phases emulsified during equilibration so that centrifugation was required to cause separation.

Not only was benzylpenicillin found to reach equilibrium at different rates in different systems but in some systems it was also found to approach equilibrium at different rates, depending on whether it passed from one phase or from the other. An interesting example of such a case was that represented by Curves 1 and 2 of Fig. 1. The net rate of "escape" of the solute from the buffer phase is slower than that from the ether phase. The reverse might have been expected, since at equilibrium the solute existed in the ether phase in 5-fold the concentration of that in the buffer phase.

In connection with this latter point Curves 3 and 4 in Fig. 1 are interesting. With this solute, *p*-hydroxybenzylpenicillin, the concentration in the buffer phase was 2.8-fold that in the ether phase at equilibrium. Yet Curves 3 and 4 are similar and show approximately equal rates of escape of the solute from either phase.

Of considerable interest to the method of counter-current distribution, particularly when applied to the investigation of unknown mixtures, might be the result obtained for the case of intentional operation of the apparatus at disequilibrium. This could be studied easily for benzylpenicillin in the

system used for Curves 1 and 2 of Fig. 1 by using something less than twenty inversions for the equilibrations during the distribution. Accordingly, a distribution was made in the standard manner with only eight inversions at each step. The result of this distribution is shown in Curve 1, Fig. 3. Had the distribution been made at complete equilibrium, the calculated distribution shown by Curve 2 would have been obtained.

These two curves suggest an interesting possibility in connection with the theoretical separation of a binary mixture. Two solutes might have iden-

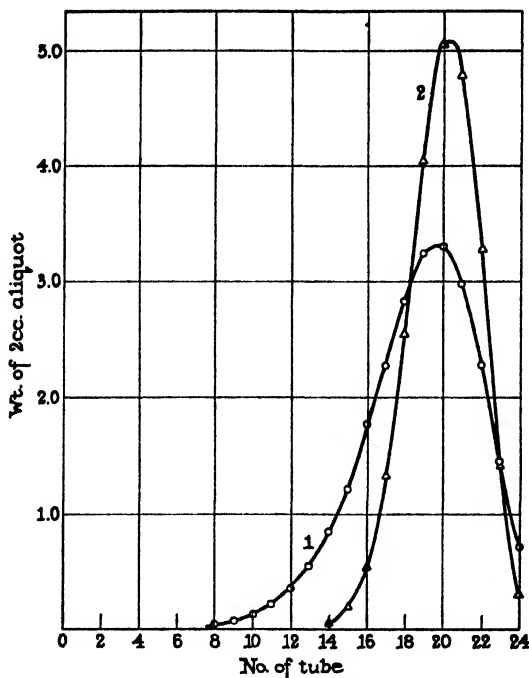


FIG. 3. Effect of disequilibrium on counter-current distribution curves. Curve 1, disequilibrium; Curve 2, calculated.

tical or nearly identical partition ratios in a particular system, yet could differ in the rate at which they would approach equilibrium from the two phases. If one behaved as shown by Curve 1 and the other behaved as Curve 2, then a partial separation could be achieved. When the partition ratios are not far apart, an improved separation could theoretically be obtained. This type of reasoning is of interest in connection with continuous fractionation columns where true equilibrium is never established at any point in the column.

A distribution was also made with benzylpenicillin at slight disequilib-

rium in the system used for the determination of Curves 1 and 2 of Fig. 2. 100 transfers were applied with twenty-five inversions at each stage. The result is shown in Curve 1, Fig. 4. The calculated curve is Curve 2. Apparently the salt concentration of this buffer was slightly higher, which accounts for the band being somewhat shifted to the right. As might be expected from Curves 1 and 2 of Fig. 2, the experimental curve is wider than the calculated one, although symmetrical in shape. The deviation in the region of Tubes 60 to 75 was due to the presence of a small percentage of penicillin of the  $\Delta^2$ -pentenyl and *n*-amyl type. That the widening of the main band was not due to a mixture of substances was established by redistributing (70 transfers) the material of Tubes 33 to 43 and comparing the

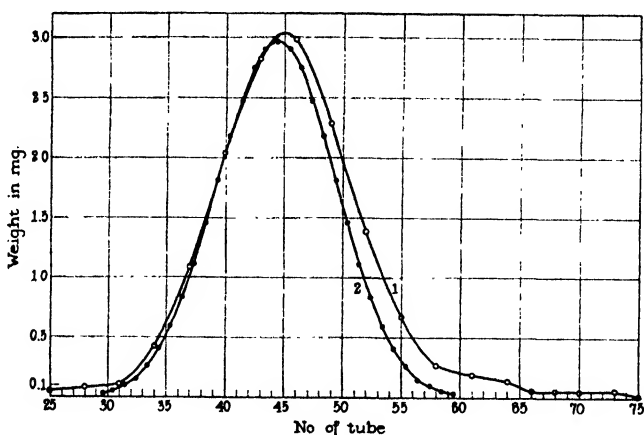


Fig. 4. Counter-current distribution curve run at slight disequilibrium, Curve 1; calculated, Curve 2.

result with a similar redistribution of the material in Tubes 48 to 58. The two curves were of the same width.

In a very interesting discussion of the theory of chromatography, Martin (5) has presented the theoretical result expected if instantaneous equilibrium should be established at every point in a column. This would imply an infinite number of stages in the column and in this case the front and rear of the band would remain sharp during migration. On the other hand, when equilibrium is not established instantaneously, as in actual experimental results, the front and rear edges of the band tend to erode more rapidly and the experimental curve obtained assumes nearly the shape of the curve of error (assuming a linear partition isotherm).

Our extraction experiments are partially analogous but differ in many respects. The experimental realization of a particular curve of error in a counter-current distribution is always possible with a linear partition iso-

therm (6) because the actual number of transfers applied is known, each transfer is nearly perfect, the solute can be placed in a single tube initially, the relative volumes of the phases in contact at each stage are known, and actual equilibrium conditions can be reached at each transfer. Any one of these variables can be changed at will in order to produce a desired effect without introducing simultaneously an unknown variation in the others.

In contrast to this, the realization of a normal curve in a packed column process such as in chromatography has a different basis. Among other reasons, it could be caused by such factors as disequilibrium or variation in the packing of the column. It would appear to be a much more complex phenomenon in which rates such as rate of flow, rate of interchange of solute, etc., must always be considered.

The authors wish to express their thanks to Dr. Vincent du Vigneaud and Dr. Frederick H. Carpenter for their interest and helpful suggestions during the course of this work.

#### SUMMARY

A method is proposed for the study of the rate of interchange of solute between two immiscible liquids in contact. The method has been applied to different crystalline penicillins in several systems. Considerable variation in the rate of interchange has been found. The implications of the study in connection with counter-current distribution have been discussed. The effect of operation of the counter-current distribution apparatus at disequilibrium has been studied.

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## DISTRIBUTION STUDIES

### XI. ISOLATION OF BENZYL PENICILLIN CONTAINING RADIOACTIVE SULFUR

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(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

(Received for publication, January 19, 1948)

In studies to be reported in *Science* by Dr. S. F. Howell and Dr. J. D. Thayer of the Venereal Disease Research Laboratory, Staten Island, New York, and Dr. L. H. Labaw of the National Institute of Health, the mold *Penicillium notatum* has been grown on a synthetic medium containing radioactive sulfur as sodium sulfate. For one experiment the culture was extracted by an organic solvent and the crude amorphous penicillin recovered by the usual lyophilization process. In another experiment adsorption was used in the recovery. The product in both cases contained radioactive sulfur.

It was of interest to isolate the penicillin from the residues in crystalline form in order to determine whether the radioactive sulfur had indeed been incorporated into the penicillin molecule. Because of the relatively small size of the amorphous samples available the counter-current distribution method (1) appeared to be the method of choice. Accordingly, at Dr. Howell's suggestion, we have undertaken the isolation of the crystalline penicillin.

For the preliminary experiment a twenty-four transfer distribution was made at a temperature of 5° with 504 mg. of the lyophilized sample in the ether-buffer system (8 cc. of each layer) which had proved the most satisfactory for the isolation of benzylpenicillin (1). The buffer was approximately 2 M phosphate at pH 4.88.

The sample was dissolved in the buffer phase, but when shaken with the ether phase a rather stable emulsion developed. 1 cc. of cold 8 M phosphoric acid was therefore added and, after shaking, the ether layer was withdrawn and used for the upper phase in Tube 0 of the distribution. No further difficulty with emulsification was experienced. After the distribution was finished, the contents of each tube were acidified with 1 cc. of 8 M phosphoric acid and shaken in order to transfer the penicillin into the ethereal layer. A 1 cc. aliquot of the ether of every alternate tube was

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then evaporated to dryness under reduced pressure and weighed. The results are shown graphically in Fig. 1, Curve 1.

The curve shows a slight elevation in the region of Tubes 8 to 14, with the apparent maximum at Tube 10. A much greater elevation occurred at the right-hand side of the graph with the maximum at Tube 23. The smaller peak occurred in the region, which would indicate benzylpenicillin or perhaps  $\Delta^2$ -pentenylpenicillin, while the much larger peak would indicate the *n*-heptylpenicillin type.

However, a study in the Venereal Disease Research Laboratory of the antibiotic activity of the different tubes restricted almost all of the activity

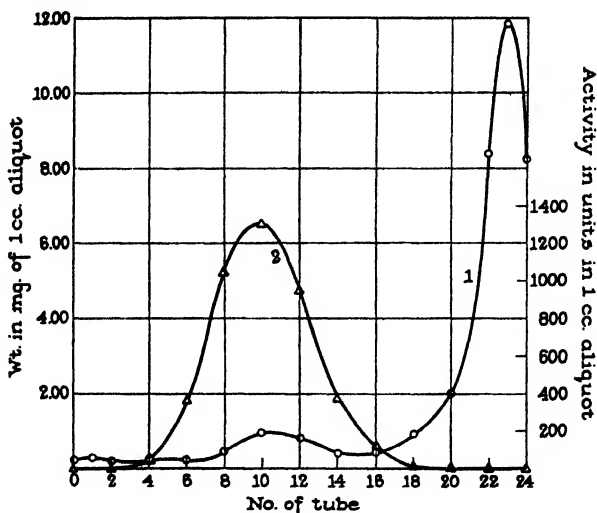


Fig. 1. Distribution of extract of penicillin culture. O, weight; Δ, biological activity.

to the region of the smaller peak where the benzylpenicillin type would occur (1). Curve 2 of Fig. 1 gives the activity per cc. of the solid material contained in each of the tubes. The distribution had accomplished a considerable concentration of the active material, since Tubes 6 to 14 comprised only 9 per cent of the weight of the initial sample.

The material in Tube 9 crystallized readily as the triethylamine salt (1) and yielded 3.7 mg. of crystals. Further, upon testing the radioactivity of the crystalline material in the laboratories of the National Institute of Health it was found to have approximately 4 times the radioactivity of the amorphous starting material.

Subsequently a larger sample (1.513 gm.) of the amorphous penicillin was fractionated by a slight variation of the first experiment. The same system was used, but instead of equal volumes of the two phases, 12 cc. of ether in each of the upper phases were employed. A total of forty-nine

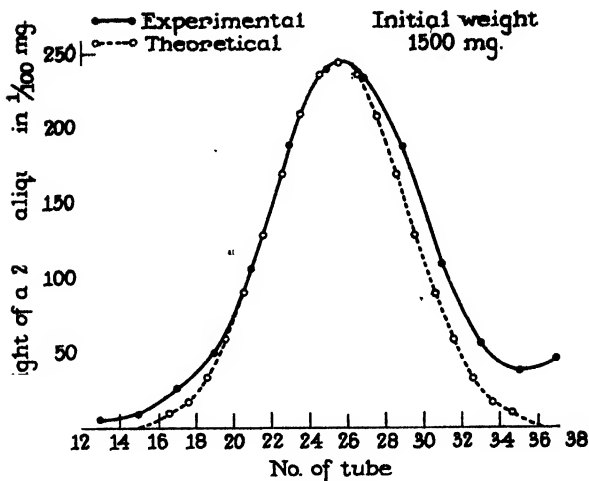


FIG. 2. Large scale isolation of penicillin G (benzyl) containing radioactive sulfur atom. ●, weight; ○, calculated curve.

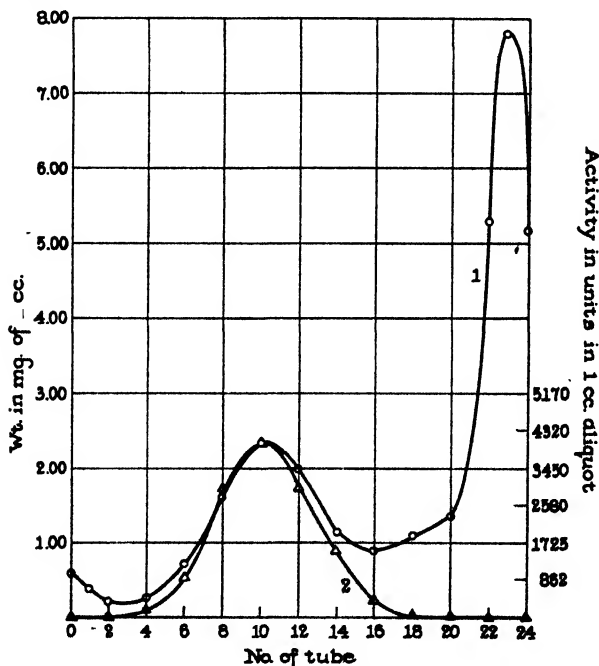


FIG. 3. Distribution of amorphous material isolated by adsorption. ○, weight; Δ, biological activity.

transfers was applied by means of the technique of alternate withdrawal (1). Fig. 2 again shows the result graphically. The majority of the inac-



tive material had been withdrawn from the apparatus before the end of the distribution and its weight does not appear on this graph. The curve refers only to the material remaining in the distribution apparatus at the end of the distribution. A calculated curve (2) is superimposed. From this curve it can be derived (1) that a small percentage of penicillins other than benzylpenicillin and perhaps inactive material is present in the right-hand side of the band.

For isolation various tubes were combined. From Fig. 2 it can be predicted that Tubes 20 to 28 inclusive are most likely the purest with respect to benzylpenicillin. These were accordingly combined and precipitated with excess triethylamine. Upon recrystallization, 68 mg. of the colorless salt were collected which melted at 140–154° (hot stage melting point).

$C_{22}H_{31}O_4SN_3$ . Calculated, C 60.65, H 7.64; found, C 60.70, H 7.78

Tubes 27 to 29 gave 27 mg. of crystalline material which melted at 131–139°.

Found, C 60.12, H 7.59

Tubes 30 to 33 gave 28 mg. of crystalline material with a slight yellow color, which melted at 119–125°. This material probably contained an appreciable amount of the  $\Delta^2$ -pentenylpenicillin and *n*-amylpenicillin types.

A similar study was made of amorphous crude penicillin isolated from the culture media with the aid of adsorption on charcoal. A distribution made exactly as in the first experiment described above is shown in Fig. 3, Curve 1. Determination of the antibiotic activity gave the result shown also in Curve 2. The pattern is very similar to Fig. 1, except that several times the amount of active penicillin were indicated.

Again assuming Tubes 7 to 11 to be the most pure with respect to benzylpenicillin, the triethylamine salt was prepared. 50 mg. of colorless crystalline benzylpenicillin salt was obtained which melted at 140–150°.

Found, C 60.54, H 7.68

The analysis reported here were made by Mr. D. Rigakos.

#### SUMMARY

The amorphous crude penicillin obtained by extraction of a culture of the mold *Penicillium notatum* grown in medium containing radioactive sulfur as sodium sulfate has been studied by the counter-current distribution process. Crystalline benzylpenicillin containing radioactive sulfur was readily isolated. Quantitative calculations could be made from the data.

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## DISTRIBUTION STUDIES

### XII. PURITY OF CRYSTALLINE PENICILLINS

By GUY T. BARRY,\* YOSHIO SATO,† AND LYMAN C. CRAIG

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

(Received for publication, January 19, 1948)

Our preliminary experience with the application of the method of counter-current distribution to the estimation of the various penicillin species present in a given preparation of the drug has previously been reported (1). Although the results were sufficient to be of considerable use, there was need for further study and improvement.

At the time the work was begun most of the penicillin preparations available on the market were amorphous and no claim was made for chemical purity. However, with the rapid advance of the technology of the production of penicillin, crystalline preparations became readily available. Unfortunately, crystallinity in itself does not guarantee purity and a method of determining the purity of such preparations was needed. Although the determination of the purity of a given preparation is fundamentally part of the problem of separation and identification, greater experimental precision and a wider experience with the particular type of compound in question are generally required. The present paper will report our further experience along this line.

Of primary importance in the application of the method of counter-current distribution to the problem of the purity of any preparation is the demonstration that a suitable system can be found in which the compound of interest will be partitioned ideally or nearly so over a practical concentration range. The value of the partition ratio should come within the optimum range, *i.e.* near 1. Such a system must furnish an environment in which the compound is stable, at least for a period of several hours time. The system must separate into its two layers readily and should offer conditions for precise analytical determination of the compound at the end of the distribution. Whether or not these conditions can be met can only be fully answered by the achievement of a distribution curve (2, 3) which is in agreement within the experimental error with the theoretical distribution calculated by means of the binomial expansion.

This latter had not been accomplished at the time of our previous publi-

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cation (1) although the type of deviation from the Bernoulli distribution observed earlier strongly indicated that it was due to impurities present in the crystalline preparations at hand. Very shortly thereafter, essentially perfect experimental distributions were obtained on a crystalline preparation of benzylpenicillin which was kindly supplied to us by the Commercial Solvents Corporation. This demonstrated beyond doubt that the discrepancies encountered earlier were due to impurities. In Fig. 1 is an example of a satisfactory twenty-four transfer distribution with a 500 mg. sample of crystalline benzylpenicillin.<sup>1</sup> The procedure and the system

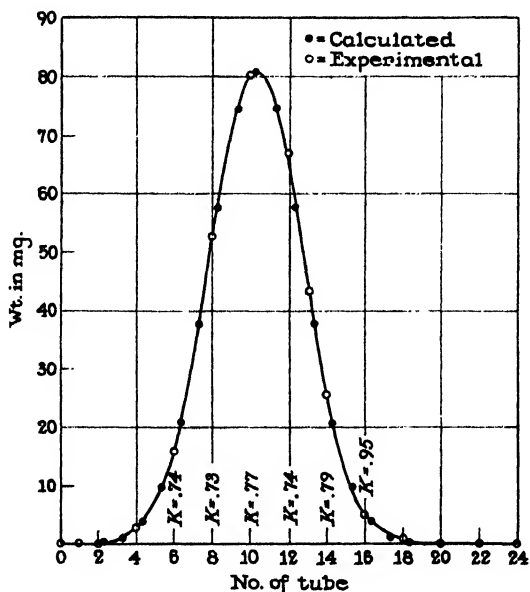


FIG. 1. Twenty-five transfer distribution of benzylpenicillin

diethyl ether-2 M phosphate at pH 4.86 described previously (1) were used for the distribution. Such a distribution was also obtained by redistribution of certain selected fractions from the preliminary distribution of a slightly impure sample.

Although the penicillins are known to be labile compounds, especially in aqueous solution, no transformation of the penicillin during the distribution at 5-6° was indicated by the results shown in Fig. 1. However, for distributions requiring greater time it would be desirable to study the stability of penicillins under the conditions of the distribution. Since many of the transformations undergone by penicillin are accompanied by changes in the

<sup>1</sup> This distribution was made with the assistance of Dr. George H. Hogeboom.

ultraviolet absorption spectra, this property was used to measure transformation rather than the more fundamental but less precisely determinable property of biological activity.

For the purpose a sample of benzylpenicillin of known extinction at wave-lengths 260 and 320  $m\mu$  was equilibrated at the temperature of the cold room (5–6°) with equal volumes of the two phases. The extinction values in the ether layer were then measured. The following day the extinction values were rechecked without equilibration. When System 3, Table I, was used, an appreciable change had not occurred. The two phases were then equilibrated and the extinctions remeasured. A small increase at 260  $m\mu$  was recorded but much more was shown at 320  $m\mu$ . Again the

TABLE I  
*Systems Studied*

System No.	Organic solvent	Phosphate buffer	K of benzylpenicillin (G)	K of $\Delta^2$ -penicillin (F)	Ratio, $\beta$
1	Ethyl acetate	1 M, pH 5.12	1.24	1.05	1.18
2	“ ether	3 “ “ 4.60	5.20	8.9	1.71
3	“ “	3 “ “ 4.93	2.54	4.00	1.57
4	Chloroform	2 “ “ 4.85	1.32	0.93	1.42
5	Furan	3 “ “ 4.93	0.34	0.50	1.47
6	Ethyl ether	2 “ “ 4.85	0.75	1.18	1.57
7	1:1 ethyl and isopropyl ethers	3 “ “ 4.93	0.96	1.72	1.80
8	Isopropyl ether	3 “ “ 4.93	0.34	0.68	1.97
9	“ “	3 “ “ 4.93	0.26*	0.61*	2.3

\* Determined at 25°.

solutions were allowed to stand until the next day and measurements made. A further shift was obtained but only after equilibration. Further progressive shifts occurred on succeeding days.

From these experiments it was apparent that a slow transformation of the penicillin was occurring in the buffer phase but apparently not in the ether phase. The free acid has been shown elsewhere (4) to be relatively stable in certain organic solvents. Other experiments in this laboratory have also supported this view.

A shift of extinction coefficient gives no quantitative estimation of the true amount of transformation occurring unless the extinction coefficient of the transformation product is known. The extinction coefficients of the transformation products could be extremely high and the value measured might thus represent only a small fraction of the original sample in terms

of weight. Fortunately, this appears to be the case for benzylpenicillin, since the total recovery in terms of weight in the main band of Fig. 1 was within the experimental error, *i.e.* a few per cent. Loss of biological activity could not be detected during such a distribution.

In distributions requiring an extended time, transformation might be expected to reach a percentage large enough to be detected by the curve. A 99 transfer run which required approximately 3-fold the time did show significant amounts of transformation products or perhaps of impurity.

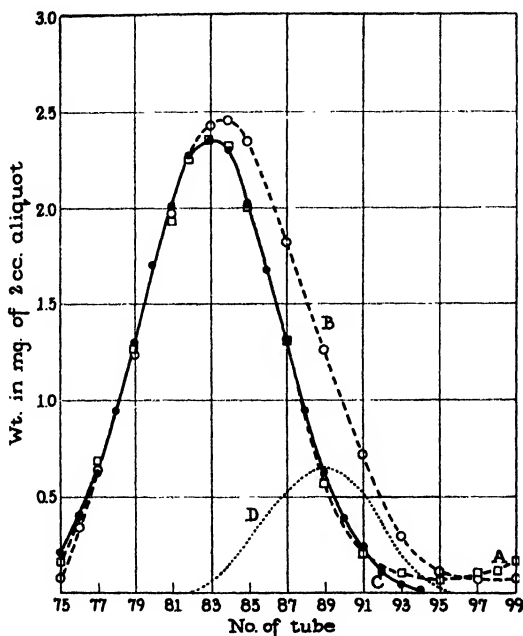


FIG. 2. 99 transfer distributions.  $\square$ , benzylpenicillin alone;  $\circ$ , benzylpenicillin +  $\Delta^2$ -pentenylpenicillin;  $\bullet$ , calculated curve.

Such a distribution is shown in Curve A, Fig. 2. The calculated curve is Curve C. The system was slightly different from that used for Fig. 1 in that 3 M buffer at pH 4.6 was used. 99 transfers were applied by the single withdrawal procedure (5). This was done by withdrawing the contents of Tube 24/0 when upper 0 has reached lower 24, and replacing it with fresh phase. After the next equilibration Tube 24/1 was withdrawn; on the next step Tube 24/2 was withdrawn, etc. The tubes withdrawn were from the slow moving side of the operation, up to Tube 74, Fig. 2, since in this system the penicillin greatly favors the ether phase. The small percentage of material in Tubes 93 to 99 had a much higher extinction coefficient and

probably represented a transformation product, although it could conceivably represent impurity.

Studies made at 25° showed a much greater rate of transformation, both in the case of individual equilibrations and for actual distributions. Thus the penicillins probably cannot ever be regarded as being *completely* stable substances when they are in contact with aqueous solutions. The rate of transformation during a distribution cannot be entirely eliminated but can be reduced until it becomes insignificant from the standpoint of the calculations. This was accomplished in the distribution of Fig. 1. It should be made clear that when transformation does occur it can be recognized, as

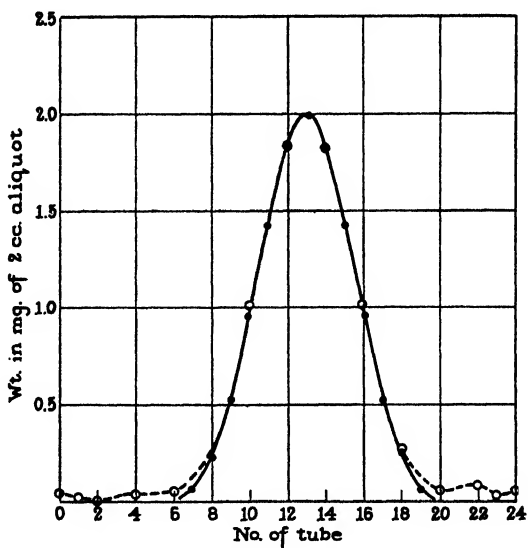


FIG. 3. Twenty-five transfer distribution of  $\Delta^2$ -pentenylpenicillin. ○, experimental; ●, calculated.

has been shown (1). Redistribution of the material in the tubes which do not show divergence should give a curve with no divergence from the calculated. This has been done with a sample of benzylpenicillin.

Other penicillin species show a different rate of transformation from benzylpenicillin. The best distribution pattern we have obtained thus far for  $\Delta^2$ -pentenylpenicillin is shown in Fig. 3. The system and procedure were the same as those employed in Fig. 1. The weight of material shown in Tubes 0 to 6 and in Tubes 20 to 24 must be due either to impurity or to transformation. The recovery represented by the main band, Tubes 7 to 19, is the equivalent of 90 per cent.

Distributions made at room temperature have not thus far permitted as

high recoveries as those made in the cold room. A representative distribution made on a mixture at  $24^{\circ}$  is excellent for comparison. This distribution, Fig. 4, was made in a 54 tube distribution apparatus in the system isopropyl ether-3 M phosphate, pH 4.93 (System 9, Table I). 71 mg. of sodium benzylpenicillin and 60 mg. of sodium  $\Delta^2$ -pentenylpenicillin were taken initially. Each tube of the machine contained 9.7 cc. of the phosphate phase and 17 cc. of the isopropyl ether phase. The recovery of benzylpenicillin calculated from the theoretical curve (No. 1) was 92 per cent, while that of  $\Delta^2$ -pentenylpenicillin (Curve 2) was 82 per cent. It would appear likely that the divergences from the theoretical were mostly

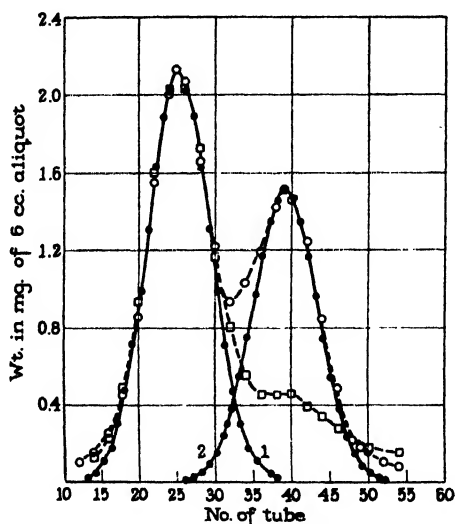


FIG. 4. 71 transfer distribution of a mixture of benzylpenicillin and  $\Delta^2$ -pentenylpenicillin. O, weight; □, extinction at 260  $m\mu$ ; ●, calculated.

due to transformation and that the  $\Delta^2$ -pentenyl species is a less stable penicillin. The recoveries calculated from Figs. 1 and 3 were 98 and 90 per cent respectively ( $5-6^{\circ}$ ).

There has been opportunity to redistribute samples of crystalline penicillins after they had been stored as the sodium salts in the cold room for a period of a year. No evidence of transformation was obtained, except with one sample of so called *n*-heptylpenicillin. This gave a different distribution pattern and was obviously undergoing progressive change. However, the original pattern had already indicated it to be a mixture. A second sample of so called *n*-heptylpenicillin did not show such a change.

Since the penicillins are inherently unstable substances, it is of interest to shorten the time required for distribution to a minimum. This could

be done by determining the minimum shaking time required in order to reach equilibrium and by transferring the phases immediately after their separation. The latter is now made possible by the new models of the distribution apparatus which are currently in operation.<sup>2</sup> The top and bottom ends of each tube of these machines are constructed from plate glass. The contents of each tube become visible when the layers have separated and this frequently shortens the total time of equilibrium, since no extra time need be allowed in order to insure the separation of the layers. The 3 minutes settling time formerly allowed empirically for each equilibration may be reduced to 1 minute or less.

The actual number of inversions required for equilibrium to be reached has been made the subject of a careful study. It has proved to be of sufficient interest to the distribution method and to extraction itself to justify a separate contribution (6) on the subject. Obviously complete equilibrium must be reached at each stage if an experimental curve is to agree with a calculated curve.

The problem of emulsification has been an annoying difficulty in the application of the distribution method to the penicillins since the very beginning. The penicillins are apparently surface-active and are capable of forming stable emulsions. In distribution work stable emulsions are to be avoided, since too great a time is required at each stage for the separation of the layers. Aside from this objection complete equilibrium is reached at each stage only with difficulty. When a transfer is made before the layers have separated completely, the relative volumes in each of the tubes become disturbed and, while fractionation is still possible, it is not as efficient as otherwise and calculated curves cannot be fitted properly.

A completely satisfactory method of overcoming the formation of troublesome emulsions has not as yet been found. However, certain changes have greatly reduced the tendency and it is possible that the addition of small amounts of surface-active agents of another type might help. In general, concentrated phosphate buffers of pH near 5.0 have not been troublesome, except in certain cases with isopropyl ether when a higher concentration of penicillin was employed. Buffers tried with higher pH could not be used because of the tendency to emulsify. Ethyl ether has given systems which separated the most rapidly. In some cases in which trouble was encountered it was overcome by increasing the volume of the upper phase. On the other hand, when an attempt was made to use a volume of the lower phase greater than that of the upper, emulsification usually occurred. In certain cases the agent which caused the emulsion was not the penicillin and could be eliminated by a single preliminary extraction. For this pur-

<sup>2</sup> Post, O., and Craig, L. C., to be published.



pose, a solution of the penicillin in the lower phase was acidified with phosphoric acid and extracted by the upper phase to be used in Tube 0 of the distribution.

Because of the emulsification difficulty most distributions were begun in individual glass tubes which could be placed in the centrifuge if necessary. Several transfers were made individually in these tubes, thereby greatly reducing the amount of penicillin in any given tube. When a separation time of 3 minutes was reached, the contents of each tube were then transferred to the corresponding tube of the machine (after shifting the upper part of the machine the required number of transfers). The remainder of the distribution was then accomplished in the usual manner.

The achievement of a perfect curve in a particular system for any given sample of a compound is unequivocal evidence for the absence of any other chemical individual, at least above a certain limiting percentage, except for the case of a chemical individual with an identical or very similar partition ratio. It can be calculated from suitable hypothetical curves that agreement between the experimental and calculated values, such as that of Fig. 1, is sufficient to eliminate anything above a few per cent which has a partition ratio greater than 1.4 or less than  $1/1.4$  times the ratio of that of the penicillin of interest. In order to detect such a possible impurity or at least to reduce greatly the probability that it is present, two approaches are at hand.

One involves the application of higher numbers of transfers and the other involves the discovery of an entirely different and more selective system for the distribution. The first simply reduces the limits of the partition ratio which an impurity might have and yet escape detection. On the other hand, the effect of the second approach cannot be predicted so precisely but will be a matter of experience. The use of both approaches in conjunction with each other is particularly important, as the following results will show.

The effect of higher numbers of transfers can be appreciated best by a consideration of hypothetical mixtures. Fig. 5 shows the effect to be expected with twenty-four transfers and equal volumes of the phases from a mixture of 90 per cent Compound A,  $K=1$ , and 10 per cent Compound B,  $K=1.4$ . The sum of Curves A + B gives Curve C.

If this mixture had been encountered as an unknown, Curve C would have been determined as a weight curve. An aliquot should have been so selected for the determination of weight that the weight of residue in the maximum tube would be at least 100-fold that of the error in weighing. Then a theoretical curve (2) would have been fitted, in this case Curve D.

Although the divergence of Curve C from Curve D appears slight at first glance, it is appreciable. At Tube 17 the determined weight is 2.6 mg.,

while the theoretical is 1.9 mg., a difference of 0.6 mg. The error in weighing could be  $\pm 0.1$  mg. Thus the divergence would be 6-fold the experimental error. Such a divergence would also be supported by the divergence at Tubes 16 and 18.

If this same mixture is subjected to 100 transfers, a much clearer picture is obtained, as shown in Fig. 6. Here the two bands are far enough separated so that nearly half the curve of Compound A on the left side is essentially

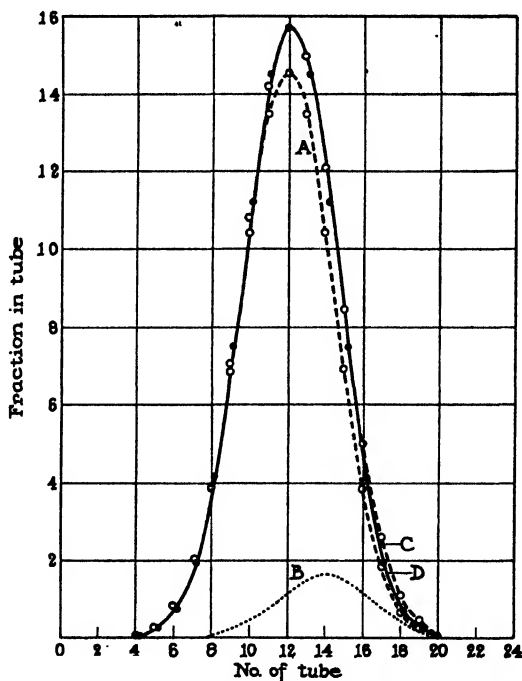


FIG. 5. Hypothetical twenty-five transfer distribution of a mixture of Compounds A and B; Curve C = sum of Curves A and B; Curve D = calculated curve matched with Curve C.

ally pure and it is therefore available for the fitting of a theoretical curve. The theoretical curve now coincides with Curve A and the difference between this curve and the one which would be experimentally determined, Curve C, would give Curve B. It is plain that for these partition ratios, 1 per cent of Compound B in Compound A could be detected.

It is now instructive to consider a mixture of substances whose partition ratios are closer together. Such a mixture could be one containing 90 per cent of Compound A with the partition ratio of 1 and 10 per cent of Compound B with the partition ratio of 1.2. Twenty-four transfers obviously

would not be sufficient to show the presence of the mixture if weights only were used for the determination of the curve. However, 100 transfers on the hypothetical mixture of Compounds A and B would give the picture shown in Fig. 7. Curve D is the theoretical curve.

Of the known penicillin species,  $\Delta^2$ -pentenylpenicillin is the one which shows a partition ratio most closely related to benzylpenicillin. Therefore a study of the partition ratios for these two species in a number of systems should be very informative in showing the effect of changing the system.

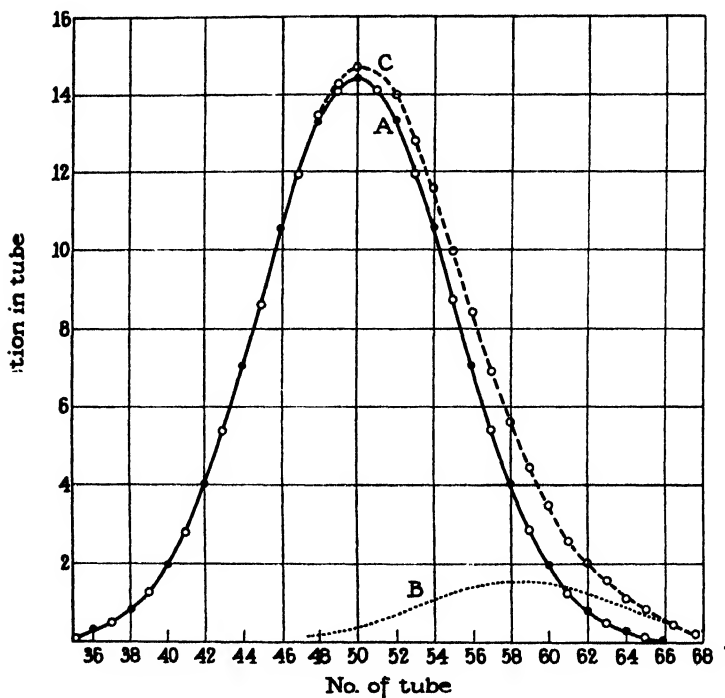


FIG. 6. Hypothetical 100 transfer distribution of a mixture of Compounds A and B; Curve C = sum of Curves A and B.

The partition ratios given in Table I were mostly calculated (2) from actual distributions but also were in agreement with individual determinations.

Except in one case the temperature was  $5^\circ \pm 1^\circ$ . A convenient numerical measure of the ease of separation of the two penicillins or of detecting one in the presence of the other is the ratio of the partition ratios shown in the last column and called  $\beta$  by previous workers (7) using extraction for fractionation purposes.

Synthetic mixtures of benzylpenicillin and  $\Delta^2$ -pentenylpenicillin have

been studied in several of the systems given in Table I. These included Systems 3, 6, 7, and 9. The result obtained with System 2 is given by Curve B in Fig. 2 and that with System 9 is shown in Fig. 4.

The interesting conclusion to be drawn from Table I is that the  $\beta$  values are in each case shifted by the change in the system. This shift is not of large magnitude, in accord with the experience of others (7, 8) for closely related substances. Irrespective of this, the practical problem of purity

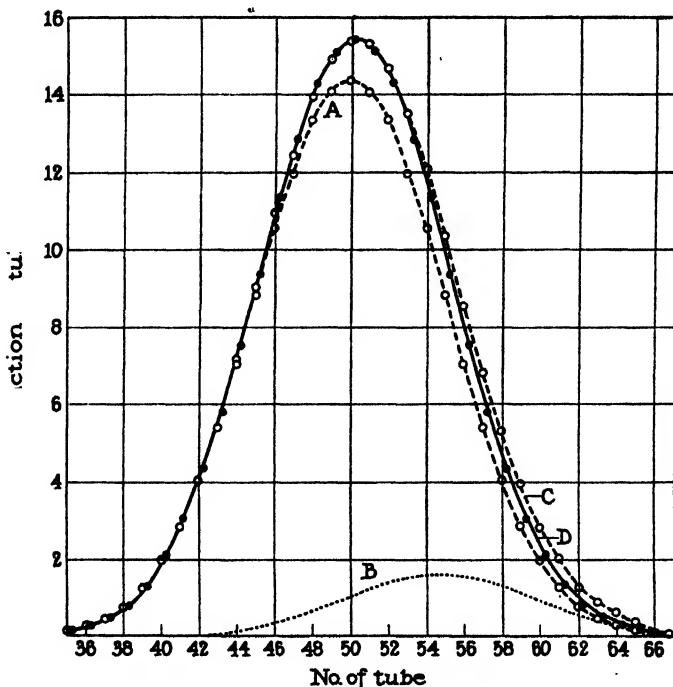


FIG. 7. Hypothetical 100 transfer distribution of a mixture of Compounds A and B; Curve C = sum of Curves A and B; Curve D = calculated curve matched with Curve C.

of the penicillin group of compounds now resolves itself into the application of a sufficient number of transfers together with adequate analytical precision so that the limiting  $\beta$  value which would permit a mixture to escape detection is small as compared to the  $\beta$  values shown in Table I.

It can be deduced from Fig. 5 that a few per cent of an impurity with a  $\beta$  value of 1.4 would escape detection with twenty-four transfers if the analytical method included weight only. Similarly, the limiting  $\beta$  value for 100 transfers (Fig. 6) would be 1.2. However, a large amount of impurity could be detected at  $\beta$  values of 1.2 and 1.1 respectively. In applying such

reasoning to the precise interpretation of the distributions obtained with penicillins it must not be forgotten that the penicillins are basically unstable and that small deviations might possibly be due to decomposition, particularly in the case of the longer runs.

In the case of Figs. 5 and 7 the sensitivity of the method is decreased because of the inability to place the theoretical curve correctly when weight only is used to determine the actual experimental curve. However, if a method of analysis specific for Compound A only in contrast to Compound B or vice versa, such as biological assay, infra-red absorption, etc., is at hand, the procedure then becomes much more sensitive and here again would permit limiting  $\beta$  values of 1.2 and 1.1 for twenty-four and 100 transfers respectively and yet permit detection of a few per cent of the one constituent.

It would appear likely that isopropyl ether-buffer is the best system for studying the purity of benzylpenicillin and of  $\Delta^2$ -pentenylpenicillin as far

TABLE II  
*Composition of Buffers*

Buffer	NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	K <sub>2</sub> HPO <sub>4</sub>	LiOH
	gm.	gm.	gm.
1 M, pH 5.12	124	17.4	
2 " " 4.85	248	34.8	
3 " " 4.60	372	52.2	
3 " " 4.93	372	52.2	7.2

as  $\beta$  values are concerned. However, isopropyl ether has the disadvantage that benzylpenicillin is of limited solubility at 5° and the system can therefore be overloaded easily. Difficulty will be encountered if more than 5 or 6 mg. of benzylpenicillin per cc. of isopropyl ether are employed at the start of the distribution. Fortunately a method is at hand for partially overcoming this difficulty. The solute can be placed in several adjoining tubes at the start instead of only one, and thus more material can be distributed without overloading either the solvent or the buffer.

This technical point has not been treated as yet in our published work but will be discussed in full in a forthcoming paper. However, it might be stated here that for higher numbers of transfers the distribution could be begun by scattering the solute equally among several adjoining tubes. If the number of tubes does not exceed more than 5 per cent of the number of transfers applied, then the broadening effect on the final curve is small. Thus for 100 transfers, the solute could be placed in up to five of the tubes initially without appreciable loss of efficiency. Distributions involving

higher numbers of transfers should always be made with proportionately more of the solute.

The buffers used in this investigation had the composition in gm. per liter given in Table II.

The authors wish to express their thanks to Dr. Vincent du Vigneaud and Dr. Frederick H. Carpenter for their interest and helpful suggestions during the course of this work.

#### SUMMARY

In connection with purity investigations of penicillins a study has been made of the procedure and conditions required in order to obtain experimentally a "calculated" distribution by the counter-current distribution technique. This has involved a study of the stability of the various penicillins in many different systems. The limitations of the method in connection with the effect of higher numbers of transfers and the effect of more selective systems have been investigated.

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# SOME PROPERTIES OF AN UNIDENTIFIED GROWTH FACTOR IN DISTILLERS' DRIED SOLUBLES\*

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Previous investigations (1) have shown the presence of an unidentified growth factor in distillers' dried solubles which is essential for the chick. The factor was shown to be distinct from the known members of vitamin B complex, streptogenin, and the cow manure factor. The object of this investigation was to ascertain whether this factor is essential for the growth of rats and to determine properties which might aid in its isolation and in its differentiation from other postulated growth factors.

## EXPERIMENTAL

In preliminary experiments designed to formulate rations which would be satisfactory for measuring unidentified growth factors with rats, it was found that neither casein extracted with ethanol nor commercial "vitamin-free" casein was satisfactory as a source of protein because of the presence of these factors.

Casein was purified in the following manner: Ethanol-extracted casein was slowly dissolved in a large quantity of water by the addition of 0.1 N sodium hydroxide to pH 7.2 with constant agitation with a powerful motor stirrer. The casein was coagulated with hydrochloric acid at pH 4.6. The supernatant liquid was withdrawn and the precipitate washed by resuspension in successive large quantities of water. This procedure was repeated several times. The purified casein was then dehydrated with methyl alcohol and acetone, dried, and ground.

A basal ration of the following composition was found satisfactory: purified casein 18 per cent, dextrin 69.5 per cent, McCollum's Salt Mixture No. 185 (2) 4 per cent, Cellu flour 2 per cent, hydrogenated cottonseed oil 5 per cent, cod liver oil 1 per cent, sulfaguanidine or succinylsulfathiazole 0.5 per cent, and, for each 100 gm. of the basal ration, a vitamin supplement consisting of riboflavin 3 mg., thiamine 3 mg., inositol 20 mg., niacin 3 mg.,

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pyridoxine 5 mg., choline 100 mg., biotin 0.01 mg., folic acid (Lederle) 0.01 mg., calcium pantothenate 0.5 mg., *p*-aminobenzoic acid 5 mg., and 2-methylnaphthoquinone 0.5 mg. To minimize the possible effect of biogenesis by microorganisms, a sulfonamide was added to the basal ration. This basal ration was used in all of the following experiments except those reported in Table I, in which folic acid was not included because it was not available at that time.

All additions to the basal ration were substituted for equivalent weights

TABLE I  
*Biological Tests for Unidentified Growth Factor in Distillers' Dried Solubles and Extracts*

Series No.	Additions to basal ration*	Average gain per wk.†
		gm.
I	None	19.1
	Distillers' dried solubles	30.3
II	None	18.9
	Extracts of solubles, autoclaved pH 12	30.9
	“ “ “ “ “ 7	31.8
	“ “ “ “ “ 2	32.1
III	None	18.4
	Distillers' dried solubles	31.3
	Extracts of solubles, ether, Extraction 1	26.2
	“ “ “ acetone, “ 2	18.3
	“ “ “ methanol, “ 3	18.2
	“ “ “ ethanol, “ 4	23.3
	“ “ “ residue	24.2

\* Extracts added in amounts equivalent to 5 per cent of solubles.

† Ten rats per lot. Least significant difference; 5 per cent level 2.4 gm., 1 per cent level 3.1 gm.

of dextrin. Each biological test was performed with eight or ten rats equally divided according to sex. The test period was 3 weeks.

The growth responses shown in Table I, Series I, indicate that the basal ration was sufficiently deficient in unidentified growth factors to test for these substances. The response with the addition of distillers' dried solubles showed that this product contained an unidentified growth factor which stimulated growth above that obtained with a ration containing the identified vitamins of the B complex.

Since the known members of vitamin B complex are soluble in water, attempts were made to extract this factor with water at widely different pH values and determine the stability under these various conditions. The distillers' dried solubles were extracted in acid, alkaline, and neutral solu-

tions under 15 pounds steam pressure for 1 hour. The extracts were filtered and aliquots equivalent to 5 per cent of the solubles were added to the rations and tested (Table I, Series II). It is apparent that all of these extracts contained the unidentified factor and that this factor is heat-stable at widely different pH values.

The possibility that this factor could be extracted with less extraneous matter by other solvents than water was investigated. Solubles were first extracted with ether in a Soxhlet extractor followed by subsequent extractions with acetone, methanol, and ethanol for 24 hour periods. The respective extracts were concentrated and equivalent amounts of each were assayed (Table I, Series III). The factor was partially extracted by ethyl ether. Acetone and methanol failed to remove any of the factor remaining in the residue, while ethanol removed part of it. Consequently, the direct extraction of the unidentified factor from solubles with organic solvents was abandoned.

Some of the chemical and physical properties of this growth factor were studied (Table II). Solubles were extracted with 0.1 N hydrochloric acid under 15 pounds steam pressure for 30 minutes. The extract was filtered and concentrated, the pH adjusted to neutral, treated with kaolin to remove part of the organic impurities, and then passed through a column of fullers' earth. The filtrate contained the active principle. The solution was treated with phosphotungstic acid in 2.5 per cent hydrochloric acid. A precipitate was formed which was filtered off and dissolved in an alkaline solution, and the phosphotungstic acid removed as the barium salt. The filtrate (Fraction F-5) from the initial phosphotungstic acid precipitation failed to stimulate growth and was therefore discarded. Fraction F-4 precipitated by the phosphotungstic acid contained the factor.

A portion of this active fraction was adjusted to pH 7. A solution of basic lead acetate was added, and the mixture stirred vigorously for 10 minutes. The resulting precipitate was collected and washed by centrifugation. The lead acetate precipitate was decomposed with ammonium sulfate. The solution was boiled for 20 minutes, cooled, and filtered. To remove any remaining traces of lead in the solutions, both solutions were treated with hydrogen sulfide, filtered, and concentrated. Assays of both solutions showed that the factor was precipitated by lead acetate (Fraction F-7), but could be only partially recovered (Fraction F-6).

Aliquots of the fraction obtained from the phosphotungstic acid precipitation (Fraction F-4) were adjusted to various pH values and passed through Tswett columns made up of the following adsorbents Darco G-60, Florisil, Lloyd's reagent, norit, Decalso, and fullers' earth. The filtrates, in amounts equivalent to 10 per cent of the solubles, were assayed for the presence of the growth stimulant.

The active substance was not adsorbed on fullers' earth and there was only a slight adsorption on Darco. However, the growth-stimulating material was adsorbed from acid solution on Florisil, Lloyd's reagent, norit, and Decalso.

TABLE II  
*Some Chemical and Physical Properties of Unidentified Growth Factor*

Fraction No.	Additions to basal ration*	Average gain per wk.†
		gm.
	None	19.0
F-1	Extract of solubles (0.1 N HCl)	29.3
F-2	F-1, treated with kaolin	30.1
F-3	F-2, through column of fullers' earth	27.6
F-4	F-3, pptd. by phosphotungstic acid	28.2
F-5	F-3, phosphotungstic acid filtrate	20.5
F-6	F-4, pptd. by lead acetate	23.7
F-7	F-4, lead acetate filtrate	20.5
F-8	F-4, through column of	
	Darco, pH 3	25.6
	“ “ 5.5	24.9
	“ “ 8.5	25.8
	Florisil, pH 1.5	23.8
	“ “ 5.5	26.7
	“ “ 8.5	28.1
	Lloyd's reagent, pH 1	21.5
	“ “ “ 5.5	27.0
	“ “ “ 8.5	25.2
	Norit A, pH 1	20.2
	“ “ “ 5.5	19.3
	“ “ “ 8.5	24.6
	Decalso, “ 1	22.7
	“ “ 5.5	26.2
	“ “ 8.5	27.3
	Fullers' earth, pH 1	26.0
	“ “ “ 5.5	28.2
	“ “ “ 8.5	27.0
F-9	Dialysate of F-1	24.5

\* Extracts of Fractions F-1 and F-9 were added in amounts equivalent to 5 per cent solubles; other extracts were added in amounts equivalent to 10 per cent solubles.

† Eight rats per lot. Least significant difference; 5 per cent level 1.8 gm., 1 per cent level 2.4 gm.

#### DISCUSSION

In a previous paper (1), it has been shown that distillers' dried solubles contain an unidentified growth factor which is essential for chicks. It has

now been shown that this product contains a factor which is essential for the rat. They are probably identical.

The factor is distinct from all the known vitamins of the B complex which were added to the rations in amounts exceeding the nutritional requirements. Since the vitamin supplement included synthetic folic acid (Led-erle), it is probably distinct from all the factors which recently have been shown to be related to or identical with folic acid (3). It is further differentiated from these factors by its stability to heat at widely different pH values, its non-adsorbability on fullers' earth, and its solubility in ether and ethanol (4-7). The solubility of this factor in ether also distinguishes it from the cow manure factor which is insoluble in ether (8). It is unlikely that this factor is streptogenin because streptogenin is present in commercial casein (9) and in purified casein (10). The limited information regarding the properties of factor X of Cary *et al.* (11) makes it impossible to attempt a differentiation at this time.

#### SUMMARY

Evidence has been presented which shows that distillers' dried solubles contain a factor essential for the growth of the rat. This factor is stable to heat, acid, and alkali. It is soluble in ether, ethanol, and water at widely different pH values. It is precipitated by phosphotungstic acid and lead acetate. It is not adsorbed on fullers' earth or Darco, but is adsorbed from acid solution on Florisil, Lloyd's reagent, norit, and Decalco.

The factor is distinct from vitamin A, vitamin D, thiamine, riboflavin, pantothenic acid, niacin, inositol, *p*-aminobenzoic acid, choline, 2-methylnaphthoquinone, pyridoxine, biotin, and folic acid. Evidence has been presented which indicates that this factor is also distinct from a number of other postulated growth factors.

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# THE MICROBIAL GROWTH INHIBITION PRODUCED BY OPTICAL ISOMERS OF $\beta$ -2-THIENYLALANINE\*

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It has already been shown that  $\beta$ -2-thienyl-DL-alanine inhibits the growth of *Saccharomyces cerevisiae* (1), *Escherichia coli* (2, 3), *Streptococcus faecalis* (3), and *Lactobacillus arabinosus* (3), and that the inhibition can in all cases be reversed by the addition of phenylalanine to the medium (1-3). In the present investigation the work has been extended to determine whether the "antiphenylalanine" effect of thienylalanine is possessed by both of its optical isomers.

The isomers of thienylalanine were prepared by resolution of the brucine salt of the N-formylthienyl-DL-alanine. They were tested for their ability to inhibit the growth of *Saccharomyces cerevisiae* and *Escherichia coli*. With both organisms it was observed that the D isomer possesses no appreciable inhibitory activity, while the L isomer has exactly double the inhibitory power of thienyl-DL-alanine.

Since the two organisms mentioned above do not require phenylalanine in the medium for growth, it seemed desirable to test the thienylalanine isomers against an organism which does require phenylalanine. For this purpose *Lactobacillus delbrueckii* LD5, which is able to use only L-phenylalanine for growth purposes (4), was selected. In the presence of an arbitrary amount of DL-phenylalanine thienyl-D-alanine had no activity, whereas thienyl-L-alanine and thienyl-DL-alanine showed inhibitory activities in the ratio 2:1 on a weight basis.<sup>1</sup>

For each of the three organisms, the same amount of DL-phenylalanine was required to reverse the inhibition produced either by a given amount of thienyl-L-alanine or by twice as much of the DL compound.

\* The authors wish to express their appreciation to the Lederle Laboratories Division, American Cyanamid Company, for a research grant that has aided greatly in this work.

<sup>1</sup> It is interesting to note that D-ethionine inhibits the growth of a strain of *Lactobacillus fermentum* which is able to use either D- or L-methionine for growth purposes. Approximately three times as much D-ethionine as DL-ethionine is required to produce the same amount of inhibition (unpublished data).

## EXPERIMENTAL

*N-Formylthienyl-DL-alanine*—Thienyl-DL-alanine was formylated by the method of Clarke, as described by du Vigneaud and Meyer (5). The product was obtained in 95 per cent yield, m.p. 173–176°.<sup>2</sup>

$C_8H_9NO_2S$ .	Calculated.	N 7.03, S 16.09
199.2	Found.	" 6.69, " 16.15

*Resolution of Formylthienyl-DL-alanine*—The formylthienyl-DL-alanine was resolved by means of the brucine salt according to the method used by Fischer and Schoeller (6) for formyl-DL-phenylalanine. 20 gm. of formylthienyl-DL-alanine and 41 gm. of dry brucine were dissolved in 240 cc. of boiling anhydrous methanol. The salt of the D isomer precipitated when the solution was cooled, and this material was recrystallized from 20 volumes of boiling methanol. The product, m.p. 127–129°, was obtained in an over-all yield of about 70 per cent. It had a rotation of  $[\alpha]_D^{21} = -38.7^\circ$  (0.2 per cent solution in water).

The original methanol filtrate was concentrated to dryness and the residue was recrystallized from 120 cc. of water. The yield of the salt of the L isomer, m.p. 121–122°, was 74 per cent;  $[\alpha]_D^{21} = -57.8^\circ$  (1.1 per cent solution in pyridine).

The brucine salts were converted to formylthienyl-D- and formylthienyl-L-alanine in yields of 80 per cent or more. The products were recrystallized from water.

The formylthienyl-D-alanine melted at 174–175° and had a rotation of  $[\alpha]_D^{21} = -76.7^\circ$  (1 per cent solution in ethanol).

$C_8H_9NO_2S$ .	Calculated.	N 7.03, S 16.09
199.2	Found.	" 6.82, " 16.52

The L isomer had a melting point identical with that of the D isomer, and the rotation of a 1 per cent solution in ethanol was  $+77.7^\circ$ .

<sup>2</sup>Calculated, N 7.03, S 16.09; found, N 6.67, S 16.26

*Thienyl-D- and -L-alanine*—The isomeric amino acids were obtained in 80 to 90 per cent yield by hydrolysis of the formyl derivatives with 1 N HBr, as described by du Vigneaud and Meyer (5). For analysis the products were recrystallized from ethanol-water. The thienyl-D-alanine decomposed at 239–244° (8° per minute) and possessed a rotation of  $[\alpha]_D^{21} = +31.6^\circ$  (1 per cent solution in water).

$C_7H_9NO_2S$ .	Calculated.	N 8.18, S 18.73
171.2	Found.	" 8.42, " 19.08

<sup>2</sup> All melting points were determined on a calibrated micro melting point apparatus.

The L isomer had a decomposition point of 239–244° (8° per minute) and a rotation of  $[\alpha]_D^{21} = -31.7^\circ$  (1 per cent solution in water).

Calculated, N 8.18, S 18.73; found, N 8.29, S 18.94

The thienylalanine isomers, as designated above, were obtained by a series of steps corresponding exactly to those by which the known isomers of phenylalanine have been separated, and they had physical properties corresponding closely to those of the phenylalanine isomers. However, it was thought desirable to obtain additional evidence for the assignment of spatial configuration. Lutz and Jirgensons have demonstrated that for amino acids of the L configuration the optical rotation changes in a positive direction with increasing acid concentration (7). The rotations of 1 per cent aqueous solutions of the thienyl-L-alanine containing 0.5, 1, 2, and 5

TABLE I

*Effect of Thienylalanine Isomers on Growth of Saccharomyces cerevisiae*

Thienyl-DL-alanine per 7 cc.	Colorimeter reading*	Thienyl-L-alanine per 7 cc.	Colorimeter reading	Thienyl-D-alanine per 7 cc.	Colorimeter reading
$\gamma$		$\gamma$		$\gamma$	
100	7	50	5	800	88
60	11	30	8	500	89
40	19	20	14	200	83
20	37	10	33	100	84
15	48	7.5	44	0	88
10	64	5	51		
0	88	0	88		

\* Klett-Summerson photoelectric colorimeter.

equivalents of HCl were  $-24.5^\circ$ ,  $-18.5^\circ$ ,  $-16.1^\circ$ , and  $-13.3^\circ$ , respectively. This afforded confirmatory evidence that the compound tested had the L configuration.

*Inhibition of Growth of Saccharomyces cerevisiae, Fleischmann Strain 139*—The medium and procedure used in the yeast growth experiments were the same as those described previously (1). Yeast growth was measured turbidimetrically with a Klett-Summerson photoelectric colorimeter with Filter 42. The inhibitory effects of the thienylalanine isomers are summarized in Table I. It will be noted that half as much thienyl-L-alanine as thienyl-DL-alanine was required to produce the same amount of inhibition. Data showing the nullification of thienylalanine inhibition of yeast growth by DL-phenylalanine are given in Table II.

*Inhibition of Growth of Escherichia coli*—The organism, procedure, and medium used in these experiments have been described previously (3).



Data from typical inhibition and nullification experiments are given in Tables III and IV. As in the case of yeast, the same degree of inhibition is achieved by a given amount of thienyl-L-alanine as by twice as much thienyl-DL-alanine.

*Inhibition of Growth of Lactobacillus delbrueckii LD5<sup>a</sup>*—The medium used

TABLE II  
*Nullification by Phenylalanine of Thienylalanine Inhibition of Growth of Saccharomyces cerevisiae*

DL-Phenylalanine per 7 cc.	Plus 50 $\gamma$ thienyl-L-alanine per 7 cc.	Plus 100 $\gamma$ thienyl-DL-alanine per 7 cc.
	Colorimeter readings*	
$\gamma$		
800	88	88
600	95	93
400	98	94
300	93	90
200	76	78
100	45	48
50	24	27
0	12	12

\* The control tubes containing no phenylalanine or thienylalanine gave colorimeter readings of 108.

TABLE III  
*Effect of Thienylalanine Isomers on Growth of Escherichia coli*

Thienyl-DL-alanine per 6 cc.	Colorimeter reading	Thienyl-L-alanine per 6 cc.	Colorimeter reading	Thienyl-D-alanine per 6 cc.	Colorimeter reading
$\gamma$		$\gamma$		$\gamma$	
3	8	1.5	7	1000	98
2.5	19	1.25	18	600	99
2.0	38	1.0	45	400	99
1.75	60	0.88	60	200	96
1.5	76	0.75	78	100	96
1.25	85	0.63	86	0	100
1.0	91	0.5	89		
0	100	0	100		

for these experiments was that described by Stokes, Gunness, Dwyer, and Caswell (4), with the exception that phenylalanine and norleucine were

<sup>a</sup> American Type Culture Collection No. 9595. Evidence has recently been presented by Rogosa (8) and Dunn and coworkers (9) to show that this organism is a variety of *Lactobacillus casei*.

omitted and pyridoxine hydrochloride was substituted for pyridoxamine. The vitamins were all dissolved in one stock solution which was renewed at least once a month. Also a stock solution was prepared containing most of the amino acids with each in a concentration of 5 mg. per cc.

TABLE IV  
*Nullification by Phenylalanine of Thienylalanine Inhibition of Growth of Escherichia coli*

DL-Phenylalanine per 6 cc.	Plus 5 $\gamma$ thienyl-DL-alanine per 6 cc.	Plus 2.5 $\gamma$ thienyl-L-alanine per 6 cc.
	Colorimeter readings*	
$\gamma$		
100	87	87
50	82	81
20	79	81
10	77	75
5	68	68
3	60	56
2	44	50
1	40	39
0.5	27	27
0	15	4

\* The control tubes containing no phenylalanine or thienylalanine gave colorimeter readings of 87 to 89.

TABLE V  
*Effect of Thienylalanine Isomers on Growth of Lactobacillus delbrueckii*

Thienyl-DL-alanine per 6 cc.	Colorimeter reading	Thienyl-L-alanine per 6 cc.	Colorimeter reading	Thienyl-D-alanine per 6 cc.	Colorimeter reading
$\gamma$		$\gamma$		$\gamma$	
1200	33	600	32	5000	112
1000	39	500	41	2500	105
600	56	300	58	1000	104
400	72	200	72	500	104
300	82	150	81	0	100
200	91	100	91		
100	103	50	101		
0	100	0	101		

The compounds to be tested were placed in 16  $\times$  125 mm. Pyrex test-tubes in a total volume of 1 cc. of water, and 5 cc. of the above medium were added. Cells from a 20 to 24 hour culture of the organism, grown in the inoculum medium of McMahan and Snell (10), were centrifuged,

washed, and resuspended in 20 cc. of saline. 1 drop of this suspension was used to inoculate each tube. Growth was estimated turbidimetrically after incubation for 40 hours at 37°, with a Klett-Summerson photoelectric colorimeter with Filter 66.

40  $\gamma$  of DL-phenylalanine were added to each of the tubes containing the amounts of thienylalanine noted in Table V. In the absence of thienylalanine this amount of DL-phenylalanine produced about 75 per cent of the maximum growth obtainable with an optimal amount of phenylalanine. The results indicate that, as in the case of the other organisms, thienyl-L-alanine and thienyl-DL-alanine show the same inhibitory activity on the basis of the amount of L isomer present, whereas thienyl-D-alanine shows no activity.

#### SUMMARY

The resolution of  $\beta$ -2-thienyl-DL-alanine has been described and the effect of the two enantiomorphs on the growth of *Saccharomyces cerevisiae*, *Escherichia coli*, and *Lactobacillus delbrueckii* LD5 has been reported. The thienyl-L-alanine caused inhibition of the growth of each microorganism which could be counteracted by the addition of phenylalanine. The D isomer had no inhibitory activity.

The authors are indebted to Dr. Julian R. Rachele and Miss Josephine E. Tietzman of this laboratory for the microanalytical work.

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# STUDIES ON CHOLINESTERASE\*

## V. KINETICS OF THE ENZYME INHIBITION

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The investigations which followed the discovery of diisopropyl fluorophosphate (DFP) posed two distinct problems: (1) whether the high toxicity of this compound must be attributed exclusively to the reaction with cholinesterase, (2) whether cholinesterase activity is essential for conduction.

Many difficulties and apparent contradictions had to be overcome before the inseparable association of the toxic signs of DFP poisoning with the inhibition of cholinesterase became evident. The ability to reverse the block of conduction in nerves exposed to DFP appeared incompatible with the irreversible inactivation of cholinesterase which was assumed to be instantaneous. It could, however, be shown that the irreversible inactivation of cholinesterase by DFP is a slow process dependent upon a number of controllable factors and, furthermore, that the inactivation of the enzyme is paralleled in every respect by the abolition of the electrical activity of nerves (1-4). The observation that conduction is possible in complete absence of cholinesterase was shown to be based on inadequate techniques (5). It is true that in some nerves as much as 90 per cent of the cholinesterase may be inactivated without impairing conduction, but this indicates only that the enzyme may be present in excess (10 times); the remaining 10 per cent is essential and cannot be inactivated without impairing conduction. The amount metabolized by this fraction is in the range to be expected on the basis of thermodynamic data (6). The relatively high concentration of DFP required to block conduction in nerves exposed to the compound could be explained by the observation that only a fraction of the DFP outside (less than 0.5 per cent) is found inside the nerve. Much of the highly lipid-soluble compound is apparently retained by the lipid membrane (5). Finally, it could be shown that the death

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following injections of DFP coincides with the inactivation of cholinesterase (6).

DFP is one of the most specific and most powerful enzyme inhibitors known (7). Although its toxicity must be referred to this single chemical reaction, the investigations mentioned have demonstrated that the effectiveness of a drug depends on a great variety of factors. Some of these factors were found in experiments on isolated nerves or on the whole animal; others, in *in vitro* experiments.

Some kinetic aspects of the enzyme inhibition of DFP have now been studied in order to determine whether or not additional factors have to be considered in the interpretation of the mechanism of DFP action. Such an analysis has been made possible by the availability of a virtually pure cholinesterase preparation, showing only one component in the analytical ultracentrifuge run. The results of these observations will be described in this paper.

### Methods

Cholinesterase was prepared by fractional ammonium sulfate precipitation from the electric tissue of *Electrophorus electricus* as recently described (8). About 50 cc. of a solution were obtained, capable of splitting 2500 gm. of acetylcholine per hour. 1 mg. of protein could hydrolyze 20 gm. of acetylcholine per hour. The enzyme solution was dialyzed against distilled water for 9 days, then divided into five equal portions and lyophilized.<sup>1</sup> A fine white powder was obtained and kept for several months in the refrigerator in sealed ampuls. One tube of lyophilized cholinesterase was dissolved in 30 cc. of solution containing the following salts: 0.1 M NaCl, 0.01 M MgCl<sub>2</sub>, 0.015 M phosphate buffer, pH 7.4. The solution could hydrolyze approximately 500 gm. of acetylcholine per hour, which indicated that the process of lyophilization and storage of the powder for several months in the ice box had no effect on the activity of the enzyme.

The enzyme solution was centrifuged at 36,000 R.P.M. for 1 hour at about 9°.<sup>2</sup> The pellet was redissolved in 4 cc. of phosphate buffer described above. Since there were some undissolved particles, the solution was centrifuged and the residue washed twice with 3 cc. of buffer. 1 cc. was capable of splitting 24.4 gm. of acetylcholine per hour. More than half of the enzyme activity was lost by the procedure. We are unable to account for this loss, since, in previous experiments, ultracentrifugation did not affect the activity under similar conditions. In the solution obtained, 1 mg. of protein was capable of hydrolyzing about 60 gm. of

<sup>1</sup> We are greatly indebted to Dr. J. A. Aeschlimann, Hoffmann-La Roche, Inc., in whose department the lyophilization was carried out.

<sup>2</sup> We are greatly obliged to Dr. K. G. Stern for the ultracentrifuge run.

acetylcholine per hour, which is close to the highest degree of purity obtained previously (8). This enzyme solution has been used in all experiments described in this paper.

The incubation at 10° and the manometric determinations of the cholinesterase activity at this temperature were carried out in a refrigerated Warburg bath.<sup>3</sup>

### Results

*Optimal Incubation Period*—"For the study of the kinetics of the inhibition of an enzyme, the possibility of working with a wide range of enzyme concentration is a great advantage. Rather high enzyme concentrations appeared advisable for determining whether the effect of DFP on the enzyme occurs on a mole to mole basis. The enzyme concentration of the preparation available was  $1.4 \times 10^{-7}$  M, calculated on the assumption of a molecular weight of about 3,000,000. This figure is based on the sedimentation rate in the analytical ultracentrifuge run (8). The concentration appeared to be proper for the investigations planned.

At first, an incubation period had to be found which offered the optimal condition. Since the inhibition of the enzyme is progressively irreversible, a prolonged incubation period would lead to the destruction of the greatest part of the enzyme, whereas the effect during a short incubation period might be too small for accurate estimation. To 0.5 cc. of the enzyme solution, 0.5 cc. of a solution was added of which the DFP concentration was  $3 \times 10^{-6}$  M. The degree of inhibition was determined at varying periods of incubation by removal of an aliquot part of the solution and dilution to 5000 times its original volume. By this dilution, the concentration of DFP falls far below its inhibitory range and that fraction of the enzyme which had not been irreversibly inactivated could be determined manometrically. As may be seen from the data of Fig. 1, at 23° inactivation of about 50 per cent is obtained after an incubation period of 150 minutes. During this period, the percentage inactivation rises at a rather high rate, whereas later the increase progresses more slowly. After 300 minutes incubation, the fraction of enzyme inactivated had risen to 66 per cent.

The experiments were repeated at 10°. The percentage inhibition, as may be seen from the data of Fig. 1, was slightly smaller than at 23°. The shape of the curve was, however, about the same. After 150 minutes of incubation, 39 per cent of the enzyme was inactivated, after 300 minutes about 55 per cent. At first glance, it may seem surprising that the difference between 10° and 23° is so small. But obviously, two antagonistic

<sup>3</sup> Manufactured by the American Instrument Company.

factors are involved: at 23°, the rate of hydrolysis by active enzyme will be higher; on the other hand, the rate of inactivation of cholinesterase by DFP will also be increased at higher temperature and therefore less active enzyme will be left at the end of the incubation period. These antagonistic effects may account for the rather small difference observed.

From the data obtained, an incubation period of 150 minutes at a temperature of 10° appeared to be a favorable condition for the study of the varying factors involved and has been used for the following observations.

*Relation between Enzyme and Inhibitor Concentrations*—Having thus established the optimal incubation period, the concentration of the inhibitor

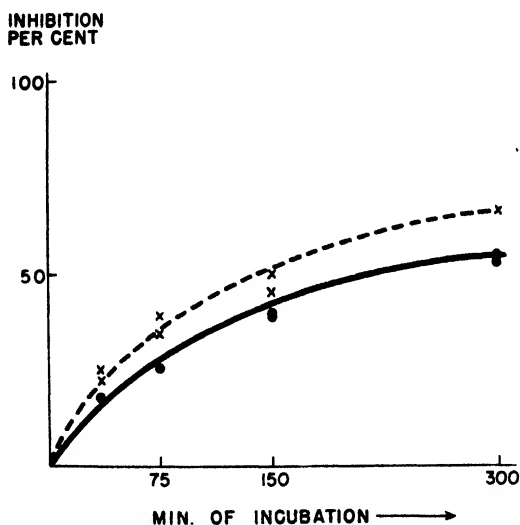


FIG. 1. Inactivation of cholinesterase by DFP at varying periods of incubation. The DFP concentration used was  $1.5 \times 10^{-6}$  M; the enzyme concentration about  $7 \times 10^{-8}$  M; × at 23°, ● at 10°.

during the incubation was varied in order to test whether or not the effect changed proportionally. As may be seen from Fig. 2, the inhibitory effect of DFP at a concentration lower than that required for 50 per cent inhibition at 150 minutes incubation decreased strictly proportionally. This suggests an inactivation of the enzyme by the inhibitor on a stoichiometric basis. The data obtained in a series of experiments were so consistent that only one set is reproduced in Fig. 2. With twice the concentration required to inactivate 50 per cent of the enzyme, the percentage inhibition rose to 86.5 per cent. The departure from strict proportionality is to be expected in that high range of inhibition.

*Excess of Inhibitor over Enzyme Concentration*—Although the strict

proportionality between increase of inhibitory effect and the increased concentration of the inhibitor suggested an action on a mole to mole basis, it appeared surprising that the concentration of the inhibitor, under the experimental condition used, was 25 times as high as the concentration of the enzyme.

In order to find out the rôle of the enzyme concentration in this connection, a series of tests was made by varying the enzyme concentration from  $10^{-7}$  to  $10^{-11}$  M. As may be seen from Fig. 3, it was found that the excess required increases rapidly with dilution. If  $pE$ , the negative log of the molar concentration of the enzyme, is plotted against the log of the ratio

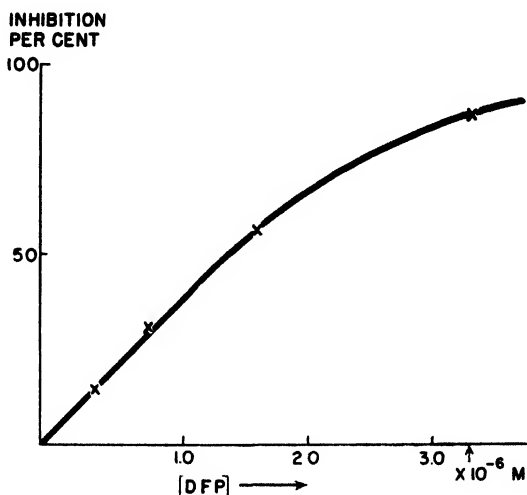


Fig. 2. Relationship between the percentage of enzyme inhibition and the concentration of DFP. Up to 50 per cent, the inhibition increases strictly proportionally to the increase of DFP concentration, suggesting a reaction on a mole to mole basis.

of inhibitor concentration,  $[I]$ , to enzyme concentration,  $[E]$ , a straight line is obtained. Whereas the excess of molecules of inhibitor over molecules of enzyme is 25 times in the highest enzyme concentration used, more than 100,000 molecules of inhibitor are necessary for each molecule of enzyme in the lowest concentration tested in order to obtain the 50 per cent inactivation.

It appears likely that the line of Fig. 3 remains straight at concentrations higher than those tested. In that case, extrapolation to the point  $\log [I]/[E] = 1$  would give a value of approximately 6. At this concentration, 1 cc. of enzyme would hydrolyze between 350 and 400 gm. of acetylcholine per hour. Such a concentration of the enzyme may be obtained. In earlier experiments, an enzyme solution was prepared, 1 cc. of which was



capable of hydrolyzing 500 gm. of acetylcholine per hour. Whether such a concentration may occur in nature is at present difficult to decide. The highest activities found for electric tissue of the eel and the head ganglion of squid corresponded to a hydrolytic power of 6 to 7 gm. of acetylcholine per gm. of fresh tissue per hour. Since it is known that the enzyme is concentrated exclusively in the neuronal surface, it is possible that if the active membrane is a layer only a few molecules thick the concentration there may be of this order of magnitude or even higher. Independent of this physiological problem, it would be of interest for the study of the kinetics of the enzyme inhibition to test the inhibitory effect in such ex-

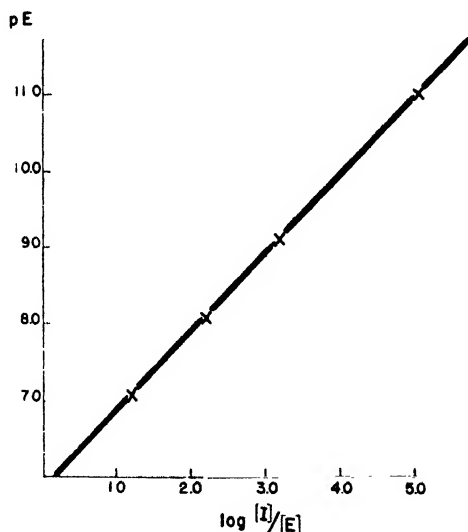


FIG. 3. Excess of DFP required for varying enzyme concentrations.  $pE$ , the negative log of the molar concentration of the enzyme, is plotted against the log of the ratio of inhibitor concentration,  $[I]$ , to the enzyme concentration,  $[E]$ .

treme concentrations. However, for the time being, no such preparation is on hand and the experiment must be postponed for later studies.

*Difference between DFP, Eserine, and Prostigmine*—The fundamental difference of the chemical reaction between cholinesterase and DFP as compared with that of other known inhibitors is obviously the progressive irreversibility. It appeared worthwhile, however, to investigate whether or not there are additional variables on which the effect of these two types of inhibitors may depend.

Since the reaction between prostigmine and cholinesterase is totally reversible, an incubation with a high enzyme concentration and subsequent dilution of several thousand times, as has been used in the case of DFP,

should lead to complete inactivation and, therefore, have no effect. This has been checked and confirmed. In experiments with diluted solutions, the immediate effect of prostigmine and eserine is in striking contrast to the slowly increasing action of DFP. As may be seen in Fig. 4, incubation with eserine for varying periods of time, up to 150 minutes, does not alter the degree of inhibition. The same has been found with prostigmine. The percentage of inhibition produced by DFP rises continuously. In this case, the inhibition represents the total effect, the reversible as well

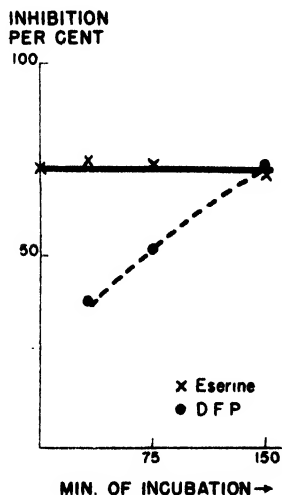


FIG. 4

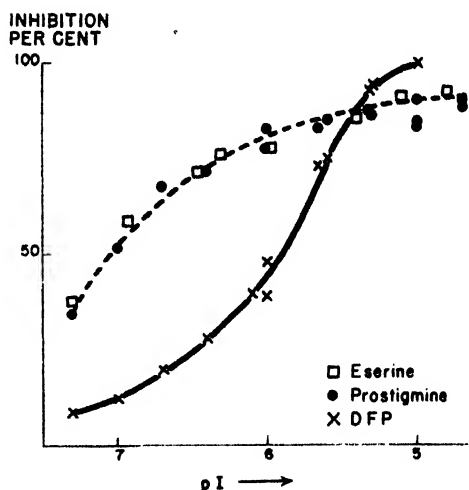


FIG. 5

FIG. 4. Difference between the effect of incubation on cholinesterase inhibition by DFP and by eserine. The prostigmine effect, like that of eserine, is unaffected by incubation. The DFP concentration, as well as that of eserine, was  $1 \times 10^{-6}$  M.

FIG. 5. Effectiveness of the inhibition of cholinesterase by DFP and the alkaloids prostigmine and eserine at varying inhibitory concentrations. Inhibition in per cent plotted against  $pI$ , the negative log of the molar concentration of the inhibitors. In the experiments with DFP, the enzyme was incubated for 150 minutes with the inhibitor before the determination. No incubation time was used in the experiments with the alkaloids.

as the irreversible part, since, at the end of the incubation period, the enzyme activity has been tested without dilution. Whereas, for short periods of contact, prostigmine and eserine are, at the same concentration, stronger inhibitors than DFP, the effect becomes equal to that produced by DFP after 150 minutes incubation and will be stronger than that of the two alkaloids if the enzyme remains in contact with the inhibitor for still longer periods of time. It is obvious that this factor will influence the toxicity *in vivo*. In warm blooded animals, i.e. at  $37^\circ$ , the reaction rate

between DFP and enzyme will be much higher than that observed at the low temperature used in these experiments. But for short periods of time, a considerable difference may be expected between the two types of inhibitors.

In another respect, the experiments have revealed a significant difference. In low concentrations, at  $10^{-6}$  to  $10^{-7}$  M, prostigmine and eserine have a much stronger effect than DFP, even after 150 minutes incubation. However, with increasing concentration, the effectiveness of DFP increases markedly and at about  $3 \times 10^{-5}$  M, it surpasses that of the two alkaloids. Fig. 5 shows the inhibitory effects obtained by varying the inhibitor concentration. These data were obtained with a constant incubation period of 150 minutes at  $10^{\circ}$ . Since the latter factor has no significant effect on the inhibitory action of the alkaloids, its change would influence only the DFP curve. At a given concentration, therefore, significant differences in effect may be produced by merely altering the incubation time.

#### DISCUSSION

Two essential facts emerge from the studies of kinetics of cholinesterase inhibition by DFP. First, the enzyme inhibition by this compound is a stoichiometric reaction. Since the inhibitory effect is produced rapidly, whereas the irreversible reaction requires a certain period of time, it appears likely that, at first, a loose addition complex is formed between enzyme and inhibitor. However, a chemical reaction, which cannot be easily reversed, soon develops between the active group of the enzyme and the DFP molecules. The rate of this reaction depends upon the temperature, the  $Q_{10}$  being approximately 2 as was found previously (4).

The second fact is the large excess of inhibitor required at low enzyme concentrations. Since the reaction probably occurs on a mole to mole basis, this large excess may indicate a low affinity for DFP. It may be suggested that the inhibitor molecule is rather small when compared with the enzyme and therefore the probability of collision with the active group is smaller in more dilute solutions. But the difference in size between the enzyme molecules and the alkaloids is nearly as great as in the case of DFP and yet the percentage inhibition is greater for the former and the reaction faster. Since we do not know the nature of the active groups and whether they are actually identical in both types of enzyme inhibition, the problem requires further investigation.

Another result of the observations is the difference between the kinetics of the inhibition by the alkaloids and by DFP. Besides the smaller reaction time just mentioned, the fact that, with a given concentration, the effectiveness of the DFP inhibition surpasses that of the alkaloids is of

great physiological interest, since the crossing of the two curves occurs in a range most important for the toxic effect. Cholinesterase, like all enzymes, is present in considerable excess; it was found to be 10 to 12 times in frog sciatic nerve, and 5 to 6 times in the abdominal chain of lobster (1, 5). It may vary in different species and even in different tissues of the same species and may not be always constant. Let us assume a range from 3 to 20 times. The toxic effects may then be expected to start after inactivation of between 66 and 95 per cent of the enzyme. In this range, the inhibition by DFP increases rapidly and approaches completion, whereas that by the alkaloids follows a more asymptotic type of curve. The interval between the onset of the first signs of intoxication by DFP and the completion of its action will therefore be rather small. In the event that a vital center is involved, the range between an inactive dose and a lethal one will be narrow.

The most striking result of these investigations is the demonstration of the innumerable factors upon which the effectiveness of the enzyme inhibition depends *in vitro*. A still greater variety of factors has an essential rôle determining the effect on cells and organs *in vivo*, such as circulation, permeability of different membranes, rate of penetration, etc. Consequently, the toxic signs show a nearly unlimited number of variations and it is not surprising that some investigators have been misled into believing in different mechanisms and in an undefined general toxic effect of DFP. The experiments lend, however, further support to the conclusion derived from the previous observations that the toxicity of DFP must be attributed exclusively to the inactivation of cholinesterase. The great variety of toxic symptoms is obviously not due to a multitude of chemical reactions but to the great number of variables which have been demonstrated to influence the course of this single reaction.

#### SUMMARY

Some kinetic aspects of cholinesterase inactivation by DFP have been studied and compared with the inhibitory effect of alkaloids. A highly purified enzyme preparation was available in which 1 mg. of protein was capable of hydrolyzing 60 gm. of acetylcholine per hour.

1. The rate of inactivation for varying periods of incubation has been measured. Under the conditions used, about one-half of the enzyme was inactivated after 150 minutes incubation. The rate of inactivation then decreased considerably.

2. Only a small difference in the rates of inactivation was found between 10° and 23°. This may be explained by the fact that the higher inactivation rate at the latter temperature is compensated for by the increase in enzyme activity from 10° to 23°. An incubation period of 150 minutes

at 10° appeared a favorable condition and was used for obtaining the data for the observations which follow.

3. With increased DFP concentration, the inactivation of the enzyme increased proportionally. The data suggest a reaction on a stoichiometric basis.

4. The reaction of DFP with the cholinesterase depends on the enzyme concentration as well as on that of the inhibitor. The greater the dilution of the enzyme, the higher is the excess of DFP required. If  $pE$ , the negative log of the molecular concentration of the enzyme, is plotted against the log of the ratio of inhibitor concentration,  $[I]$ , to that of the enzyme,  $[E]$ , a straight line is obtained. In the concentration generally used for manometric determinations, an excess of more than 100,000 molecules of inhibitor is necessary per molecule of enzyme.

The fundamental difference of the inhibition of cholinesterase by DFP as compared with that by the alkaloids prostigmine and eserine is the irreversible nature of the former. Additional differences, however, were observed.

5. Incubation at periods up to 150 minutes did not affect the inhibitory action of the alkaloids, in contrast to that of DFP.

6. In lower concentrations the alkaloids have a stronger effect than DFP, even if the former are tested without and the latter after 150 minutes incubation. In higher concentrations, the DFP inhibition becomes more effective and rapidly approaches completion, whereas that of the alkaloids follows a more asymptotic type of curve.

The experiments offer additional support for the conclusion that the great variety of toxic symptoms by DFP poisoning must not be attributed to a multitude of chemical reactions but to the great number of variables influencing the course of the single reaction with cholinesterase.

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# THE REACTION BETWEEN THIOBARBITURIC ACID AND THE OXIDATION PRODUCTS OF CERTAIN LIPIDES

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In 1944 Kohn and Liversedge (1) showed that tissue suspensions or slices incubated aerobically produce a compound which will condense with either *p*-aminobenzoic acid (PAB) to give a yellow color or with thiobarbituric acid to give an orange-red color. Brain is the most active tissue, liver next, and other tissues show some activity. Subsequently, Bernheim *et al.* (2) found that washed brain suspension boiled for 10 minutes and then incubated aerobically with small amounts of ascorbic acid takes up oxygen and produces the substance which condenses with PAB or thiobarbituric acid. Apparently, in the presence of ascorbic acid something is split from the brain protein. Elliott and Libet (3) have shown that phospholipides increase the oxygen uptake of brain suspensions and that this effect is increased by ascorbic acid. Accordingly, we prepared and purified lecithin from brain and also from eggs, and, after incubating this lecithin with ascorbic acid, obtained colors with PAB and thiobarbituric acid. The absorption spectra of both are identical with those reported by Kohn and Liversedge. It thus seems that ascorbic acid catalyzes the oxidation of lecithin, either free or combined with tissue protein, and that this oxidation can be followed quantitatively by the use of either one of the reagents

## EXPERIMENTAL

Egg lecithin was prepared by extracting yolks with an alcohol-ether mixture, filtering, evaporating to a small volume, and precipitating with acetone. The precipitate was dissolved in petroleum ether and reprecipitated with acetone. This process was repeated three to five times. Brain lecithin was prepared from rat brain by first desiccating the ground tissue with acetone and then extracting it with petroleum ether and precipitating with acetone. Cephalin, prepared according to the method of Folch (4), was kindly supplied by Dr. J. H. Ferguson, lysolecithin by Dr. H. B. Collier, and sphingomyelin and sphingosine by Dr. H. E. Carter.

The experiments were carried out at pH 6.0 because the pH optimum of the reaction with free lecithin is the same as that found by Kohn and Liversedge for tissue suspensions. Lecithin which had been kept under

acetone in the ice box was rapidly dried, weighed, and either shaken into an emulsion in 0.05 M sodium-potassium-phosphate buffer, pH 6.0, or dissolved in 95 per cent alcohol. The required amount was put into 50 cc. Erlenmeyer flasks with or without ascorbic acid which had been brought to the same pH. The total volume in each flask was 4.0 cc. The flasks were filled with 100 per cent oxygen and incubated with shaking in a bath at 37°. No reaction occurred in nitrogen. Heating at 100° or at 125° in the autoclave gave a negative result, presumably because the product of the reaction is destroyed at high temperatures. The instability of the product is also shown by the fact that evaporation to dryness even at low temperatures completely inhibited the reaction. After the addition of trichloroacetic acid, aliquots of the incubated material were taken, and the thiobarbituric acid reagent of Kohn and Liversedge was used to develop the color. The absorption spectra were measured in the Beckman spectrophotometer.

Most of the experiments were done with egg lecithin. As soon as it was prepared, a sample was tested with the thiobarbituric acid reagent. This reagent was used rather than PAB because it gives a somewhat more stable color, which was estimated in the Evelyn photocolormeter with a No. 540 filter. Because of the slight opalescence of lecithin solutions, controls without the addition of thiobarbituric acid were used to estimate the light absorption of the lecithin itself. When a lecithin-protein complex was used, addition of trichloroacetic acid precipitated the lecithin with the protein, which could be centrifuged off to leave a clear solution. The amount of color produced by the freshly prepared lecithin varied considerably in different preparations. Incubation of the lecithin for 2 to 3 hours at 37° in 100 per cent oxygen without ascorbic acid produced some increase in color. This also occurred if the lecithin was allowed to stand in the ice box for several weeks. Autoxidation, therefore, produces the same substance (indicated by the identity of the absorption spectrum) as oxidation in the presence of ascorbic acid. The latter catalyzes the reaction.

Table I shows the effect of varying the lecithin and ascorbic acid concentrations on the color developed after incubation. The values ( $D = (2 - \log T) \times 100$ ) are corrected from a calibration curve, for the absorption is proportional to the concentration at low concentrations only. Accurate results were difficult to obtain, because of the tendency of the lecithin to be adsorbed on glass surfaces, thus altering the amount in solution. Better proportionality was obtained with the brain protein-lecithin complex (2). When 1.0 to 2.0 mg. of lecithin was used with ascorbic acid, maximum values were obtained at the end of 2 hours incubation. Further incubation was without effect even if more ascorbic acid was added at the end of the 2 hour period. The ascorbic acid is thus not the limiting factor.

In order to determine what part of the lecithin molecule was undergoing oxidation, the following experiments were done. Incubation of choline, glycerol, or  $\beta$ -glycerophosphate with ascorbic acid gave negative results. Apparently the fatty acid part of the molecule is the point of attack. To prove this, lecithin was hydrolyzed by KOH in the usual way and the

TABLE 1

*Effect of Lecithin, Ascorbic Acid, and Fatty Acids on Color Development*

Color produced after incubation of egg lecithin with 0.2 mg. ascorbic acid for 1.5 hrs. at 37°, pH 6.0, in 100% O <sub>2</sub> ; lecithin dissolved in 95% alcohol; each vessel contained 3.8 cc. buffer and 0.2 cc. alcohol; control values without ascorbic acid have been subtracted	
Lecithin mg.	Color, $D = (2 - \log T) \times 100$
0.25	3.3
0.50	5.8
1.00	10.1
2.00	18.2
Color produced after incubation of ascorbic acid with 5.0 mg. lecithin for 3 hrs. at pH 6.0; control values without ascorbic acid have been subtracted	
Ascorbic acid in 4.0 cc.	
0.001	7.1
0.0025	7.9
0.005	16.6
0.010	20.1
0.025	24.6
0.050	35.1
0.100	33.1
Color produced after incubation of linseed oil fatty acids with 0.2 mg. ascorbic acids for 2.5 hrs. at 37°, pH 6.0, in 100% O <sub>2</sub> ; fatty acids dissolved in 95% alcohol; each vessel contained 3.8 cc. buffer and 0.2 cc. alcohol; control values without ascorbic acid have been subtracted	
Fatty acids	
0.5	1.9
1.0	2.9
2.0	7.3

fatty acids isolated. When these were suspended in buffer and incubated with ascorbic acid, positive results were obtained. In confirmation of this, highly purified linseed oil, a product of the F. Weber Company, Philadelphia, was hydrolyzed, and after incubation of the fatty acids, positive results were also obtained (Table I). The values in the absence of ascorbic acid were high, indicating that considerable autoxidation had taken place.

The double bond in the fatty acid is a probable site of the oxidation. Lysolecithin, which has no unsaturated fatty acids, gave negative results.



Sphingomyelin and sphingosine also were negative, and they contain amino and hydroxy groups; so these cannot be involved in the oxidation. Different amounts of iodine were added to a solution of lecithin which was warmed until all the iodine had disappeared, as judged by the starch test. Incubation of the iodine-treated samples showed that the color reaction was reduced more or less in proportion to the amount of iodine added (Table II). Attempts to perform this experiment with the use of Hanus' solution were unsuccessful because of the tendency for iodine to be liberated on standing after the end-point has been reached. The iodine would thus interfere with the ascorbic acid. Oleic and ricinoleic acids, both of which

TABLE II

*Effects of Adding Iodine, Semicarbazide, and Phenylhydrazine to Egg Lecithin*

The effect of adding different amounts of 0.005 N iodine to 5.0 mg. of egg lecithin before incubation and the effect of adding different amounts of semicarbazide and phenylhydrazine after incubation at 37° for 2.5 hours in 100 per cent O<sub>2</sub> at pH 6.0. The control values without ascorbic acid have been subtracted.

Reagent added	Color, $D = (2 - \log T) \times 100$
None	16.6
0.3 cc. I <sub>2</sub>	8.5
0.5 " "	2.9
0.8 " "	0.0
None	19.6
0.10 mg. semicarbazide	13.4
0.25 " "	12.0
0.50 " "	3.2
0.10 " phenylhydrazine HCl	18.5
0.50 " " "	12.4
0.75 " " "	9.4

have one double bond in the middle of the molecule, gave negative results. Therefore, this double bond is not oxidized to produce the chromogenic compound. (Cf. Bailey (5) for a discussion of the different reactivities of double bonds in fatty acids.)

The nature of the oxidation product was investigated. After incubation of lecithin, small amounts of semicarbazide or phenylhydrazine were added to the mixture before the thiobarbituric acid. Table II shows that such additions inhibited the subsequent color formation, which suggests that an aldehyde or ketone group is present. It is probable, however, that such groups are potentially rather than actually present and are only formed in the presence of the reagents, because the straight chain aldehydes or ketones tested will not condense with thiobarbituric acid to produce a

color. What probably happens is that the double bond is oxidized to form an ether or peroxide linkage, which reacts with the thiobarbituric acid (or PAB). The molecule is split by this reaction and either the longer or the shorter part combines with the reagent.

In order to determine the size of the molecule attached to the thiobarbituric acid, the colored product was isolated. After incubation of the lecithin and ascorbic acid and the addition of thiobarbituric acid, the mixture was extracted with isoamyl alcohol. The colored compound was easily extracted and the alcohol solution was washed several times with acid water. The compound was next extracted from the alcohol by dilute alkali, which was then acidified and extracted with ether to remove the excess thiobarbituric acid. It was extracted with isoamyl alcohol, which was then slowly evaporated down. The colored compound precipitated in a flocculent mass, which was centrifuged off and dried. The dry powder was thoroughly extracted with boiling ether to remove any remaining free thiobarbituric acid.

The product still had some inorganic residue but no organic phosphate, as determined after digestion with perchloric acid by the method of Fiske and Subbarow (6); so presumably the whole lecithin molecule is not combined with the thiobarbituric acid. The analysis of the compound corrected for the inorganic residue gave 43.3 per cent C, 16.7 per cent S, and 15.3 per cent N. If a 3-carbon chain with 1 oxygen atom were added to the thiobarbituric acid, the theoretical values would be 42.0 per cent C, 16.3 per cent S, and 14.4 per cent N. These values are sufficiently close to indicate that 3 carbon atoms are removed from the fatty acid. Assuming the addition of 3 carbon atoms and an oxygen, the yield of the colored compound is about 15 per cent. Recent work on the position of unsaturated bonds (7) shows that in the highly unsaturated acids (linolenic and others) a double bond is placed between groups of 3 carbon atoms.

Incubation of lecithin with  $\text{H}_2\text{O}_2$  instead of ascorbic acid gave negative results. Any excess  $\text{H}_2\text{O}_2$  which might interfere with the test was removed by catalase before the addition of thiobarbituric acid. Crystalline rat hemoglobin will, however, act catalytically for the brain preparation, lecithin, and the fatty acids from linseed oil and is a better catalyst for the last than ascorbic acid. The compound formed has the same absorption spectrum regardless of the catalyst (Fig. 1). 1.0 mg. of epinephrine inhibits the catalytic action of ascorbic acid and hemoglobin completely, and 0.5 mg. of NaCN causes partial inhibition, probably by combining with the end-product.

A few experiments were done with cephalin. The autoxidation of the preparations used was large, but ascorbic acid still showed an effect. Hemoglobin, however, does not act as a catalyst for the oxidation of

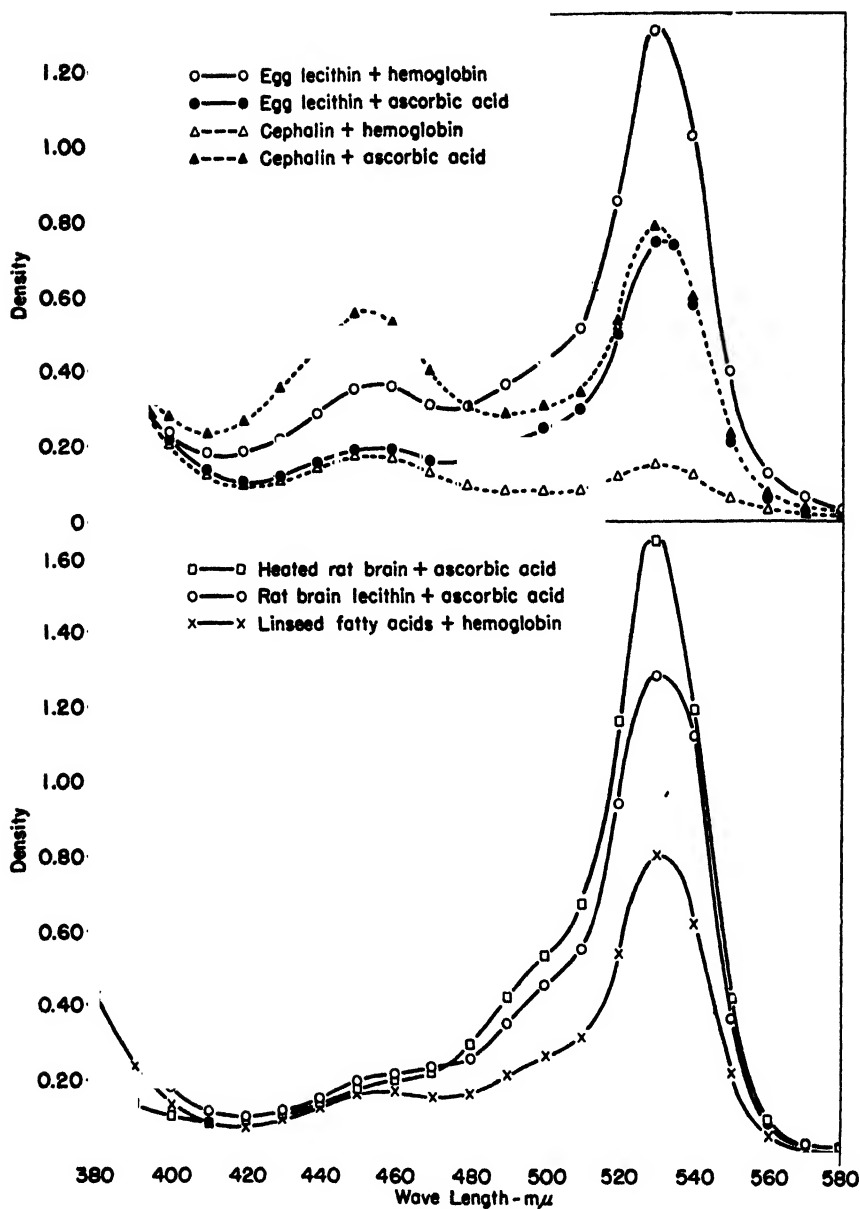


FIG. 1. The absorption spectra of the thiobarbituric acid compound formed after the oxidation of various lipides.

cephalin. In fact it even inhibits the autoxidation as well as the action of ascorbic acid. After incubation of 5.0 mg. of cephalin alone, the read-

ing was 42.4; with 0.2 mg. of ascorbic acid, 57.2; with hemoglobin, 15.2; and with ascorbic acid and hemoglobin together, 24.6. Cephalin added to hemoglobin and lecithin does not inhibit the catalytic action of the hemoglobin on lecithin. Therefore, cephalin does not inactivate the hemoglobin. The absorption spectrum of the compound made from cephalin shows a slightly more pronounced band at 450  $m\mu$  than does the compound made from lecithin (Fig. 1); otherwise they are identical. Experiments with inorganic iron and copper salts as catalysts were inconclusive, because they interfered with the thiobarbituric acid test.

#### DISCUSSION

The reaction described above, namely the oxidation of a double bond and the breaking off of a fragment containing 3 carbon atoms from the end of the chain, could occur in linolenic acid. Linolenic acid, or other similar unsaturated acids, may be formed in the oxidative metabolism of brain, liver, and kidney, and possibly other tissues as well, because, after tissue slices are incubated aerobically, strongly positive thiobarbituric acid tests are obtained. The fact that such acids have not been isolated from liver (8), for instance, may mean that they are not stored because they are rapidly oxidized. Similarly, such acids have not been reported in egg lecithin, but may be present in small amounts. It is not possible as yet to decide how the oxidation proceeds in the body; *i.e.*, whether autooxidation or catalysis by ascorbic acid or hemoglobin is responsible for the reaction. The evidence suggests, however, that oxidation of certain double bonds in a fatty acid can occur whether the fatty acid is free or bound in the lecithin molecule. The thiobarbituric acid test as well as the PAB test is a simple tool for studying this reaction in tissues under different conditions.

In 1917, Plaisance (9) showed that thiobarbituric acid added to fructose, which had been previously heated in strong acid, produced a yellow precipitate. We have prepared this compound and measured its absorption spectrum, which is completely different from the one obtained with fatty acids. It may also be mentioned that thiobarbituric acid produces a brilliant orange-red color when added to wood chips or sawdust. The color turns green in alkali, which is not true of the fatty acid color. We have not as yet been able to extract the color from the wood particles.

#### SUMMARY

1. The colors obtained by Kohn and Liversedge upon addition of either thiobarbituric or *p*-aminobenzoic acid to incubated tissues are due to a product of the oxidation of unsaturated fatty acids.

2. Egg lecithin, or fatty acids isolated from it, brain lecithin, fatty acids

from linseed oil, cephalin, and the brain protein-lecithin complex previously described give colors with these reagents after aerobic incubation with ascorbic acid. The absorption spectra of these colors are identical with those described by Kohn and Liversedge.

3. After autoxidation the same colors are produced. Ascorbic acid is thus acting as a catalyst. Crystalline hemoglobin also acts catalytically for all the lipides except cephalin. Incubation with  $H_2O_2$  gave negative results.

4. The mechanism of the reaction is discussed.

*Addendum*—After incubation, pure linoleic acid (Finer and Amend) gives only a trace of color with thiobarbituric acid. Under the same conditions pure methyl linolenate (Hormel Foundation) produces a large amount of the characteristic color. Ditertiary butyl peroxide (Shell Corporation) does not react with thiobarbituric acid to give a color.

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# ON THE INHOMOGENEITY OF COMMERCIAL HEPARIN PREPARATIONS FROM THE PHYSICOCHEMICAL POINT OF VIEW

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Heparin is known to be inhomogeneous. Jorpes found, for instance, that heparin prepared from ox liver was not a homogeneous substance. Heparin has also been obtained as a crystallized barium salt, and a divergence of opinion exists with regard to its degree of purity, homogeneity, etc. For details the reader is referred to Jorpes (1) and Chargaff (2).

Extensive physicochemical investigations have not, as far as we know, been carried out on heparin. In connection with other studies we undertook physicochemical measurements on two of the purest trade preparations of heparin, *viz.* Roche (Switzerland) and Vitrum (Sweden). Molecular weight determinations for heparin Vitrum were carried out earlier by Grönwall, Ingelman, and Mosimann (3). They reported a molecular weight of 17,000, which we have now verified. At the same time, a certain polydispersity was found which led us to a closer investigation of the questionable homogeneity of heparin.

## EXPERIMENTAL

**Electrophoresis**—Wilander found only one active component with a mobility of  $17$  to  $19 \times 10^{-5}$  sq. cm. per volt per second. Chargaff *et al.* (4) also found only one component on electrophoresis. In our electrophoretic investigation of heparin we found *two* distinct *active* components, both of which moved towards the anode. Heparin Vitrum and Roche gave the same result.

The Tiselius electrophoresis apparatus was used for this investigation (*cf.* Svensson (5)).

The experiment with heparin Vitrum was made with 1 per cent heparin solution in phosphate buffer at pH 6.8 and ionic strength 0.1. The potential gradient was 6.75 volts per cm. After electrophoresis for 1 hour a division was obtained into two components,  $\alpha$  and  $\beta$ , with mobilities of  $u_{\alpha} = 19.2 \times 10^{-5}$  and  $u_{\beta} = 16.0 \times 10^{-5}$ . The following percentage compositions could be calculated from the electrophoresis diagram:  $\alpha = 54$  per

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cent,  $\beta = 46$  per cent. The faster peak appeared more homogeneous. The slower peak tended to broaden to a certain extent (Fig. 1). After electrophoresis for a longer time, a third small component could be observed which had a mobility between those of the  $\alpha$  and  $\beta$  peaks.

After electrophoresis for 9 hours with compensation, the components were sufficiently distinct and separation could be undertaken. In one cell a pure  $\alpha$  component was obtained and in the other  $\alpha + \beta$ . The anticoagulant activity was assayed by means of the thrombin method of Jaques and Charles (6) and gave the following results:  $\alpha = 75$  per cent of the original activity,  $\alpha + \beta = 100$  per cent.

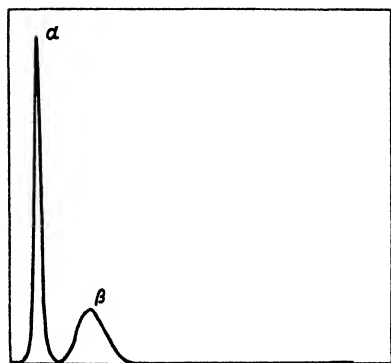


FIG. 1. Electrophoresis diagram of heparin Vitrum after 90 minutes (positive limb).

Consequently both components are active, but the  $\alpha$  component has the greater activity, since the percentage composition indicated that the components were present in the ratio 1:1.

The buffer was separated from the different fractions by dialysis, after which they were "freeze dried." Analyses for ester sulfate and hexosamine were then made on both fractions. The hexosamine content was practically the same in both fractions, but the  $\alpha$  fraction contained 9.0 per cent ester sulfate, while the  $\alpha + \beta$  fraction contained only 7.3 per cent ester sulfate. Thus, the  $\alpha$  component is definitely richer in ester sulfate than the  $\beta$  component.

When heparin Roche was examined under the same experimental conditions, we found the mobilities to be  $u_\alpha = 18.4 \times 10^{-5}$ ,  $u_\beta = 15.0 \times 10^{-5}$ , and the percentage composition  $\alpha = 50$  per cent and  $\beta = 50$  per cent. Also in this case the  $\beta$  component became broader (Fig. 2). After electrophoresis for 8 hours the experiment was stopped, the  $\alpha$  and  $\alpha + \beta$  fractions taken out, and their activities determined:  $\alpha = 55$  per cent of the original activity and  $\alpha + \beta = 100$  per cent.

Obviously heparin is characterized electrophoretically by two main components which occur in about the same proportions but have different anticoagulant properties. The composition of heparin Roche and Vitrum seems to be fairly similar.

*Adsorption Analysis*—We have also used the adsorption technique of Tiselius, further developed by Claesson (7). By means of a frontal adsorption analysis a diagram was obtained which shows a step for each component, but no definite statements can be made as to the amounts of the com-

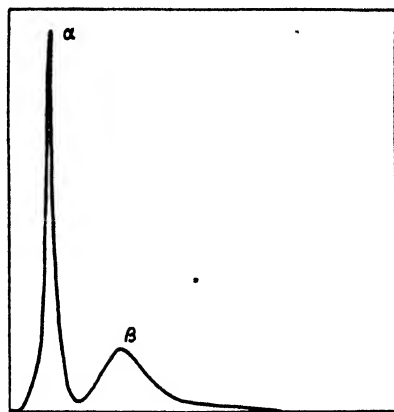


FIG. 2. Electrophoresis diagram of heparin Roche after 120 minutes (positive limb).

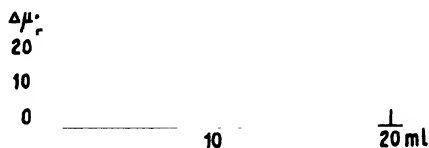


FIG. 3. Frontal adsorption analysis of an 0.20 per cent heparin Vitrum solution in water. The ordinate gives the increase in the refractive index.

ponents. The analysis of heparin Vitrum with "Carboraffin supra" (Lurgi) as the adsorbent gave a frontal analysis diagram with three components (Fig. 3). Only the first component could be assayed and its anticoagulant activity was found to be low. On the other hand, heparin Roche gave four components during a similar analysis (Fig. 4).

*Recrystallization Experiments*—It was of interest to study the effect of recrystallization on heparin, and, in particular, of the recrystallization of the barium salt in warm acetic acid according to Wolfrom *et al.* These authors reported (8, 9) that heparin Roche partly loses its activity after recrystallization, despite the fact that the sulfur content maintains its



initial value. During this process they found that amino groups were liberated from the heparin molecules.

In our experiments to recrystallize heparin Roche the results given in Table I were obtained. Recrystallization experiments were also performed with heparin Vitrum, and similar results were obtained (Table II).

Determinations of the anticoagulant effect by the thrombin method of Jaques and Charles showed a decrease in the heparin activity, although

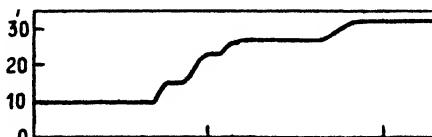


FIG. 4. Frontal adsorption analysis of an 0.20 per cent heparin Roche solution in water. The ordinate gives the increase in the refractive index.

TABLE I

*Recrystallization of Heparin Roche according to Wolfrom et Al.*

	Anticoagulant effect		Metachromacy (MacIntosh (10))		$n_D$	Ester sulfate*	Hexosamine*
	Measured as Ba salt	Calculated as Na salt†	Measured as Ba salt	Calculated as Na salt†			
	per cent	per cent	per cent	per cent		per cent	per cent
Na salt...		100		118	2.07	9.0	21
Ba " (from Na salt)	80	98	111	136	2.24	9.4	
I, recrystallized	67	82	77	94	2.37		
II, recrystallized	62	75	81	90	2.73		
III, recrystallized	56	68	82	100	2.66		
IV, recrystallized	44	54	90	110	2.51		
V, recrystallized	36	44	87	106	2.70	9.5	24.0

\* The analyses were calculated for air-dried substances as Na salts.

† Conversion factor 1484/1210 (see Jorpes (1) p. 34). An initial sodium salt was given as 100 per cent.

not quite so great as that found by Wolfrom *et al.* (8), while the degree of metachromacy determined according to MacIntosh (10) remained constant within the limits of error. Analyses showed also that the content of the ester sulfate and hexosamine did not change appreciably during five recrystallizations (Table I).

On the other hand, the ultracentrifuge measurements showed (Tables I and II) that the sedimentation constant increases continuously, and, further, at the same time the ultracentrifuge diagrams indicated that the polydispersity of the preparation also increases. An increase in the sedi-

mentation constant may be due to polymerization or other structural rearrangements of the molecule.

In order to examine this question a molecular weight determination was made on the 5 times recrystallized heparin Roche. Determination of the diffusion constant for this recrystallized heparin in the cell designed by Claesson (11) gave the value  $D_A = 7.91 \times 10^{-7}$  sq. cm. per second. By inserting the values obtained for  $S_{20}$  and  $D_A$  in Svedberg's formula for molecular weight, and by the use of the specific volume which has been obtained for commercial heparin ( $V = 0.50$ ), we found an average molecular weight of 16,600, which is in good agreement with the molecular weight determined by Grönwall *et al.* for heparin Vitrum. On the other hand, when the frictional ratio is calculated with the help of the formula (12),

$$f/f_0 = 10^{-}$$

the value 1.81 is obtained, while for the commercial heparin this factor is 2.5 (3).

TABLE II  
Recrystallization of Heparin Vitrum according to Wolfrom *et al.*

	Anticoagulant effect		Metachromacy (MacIntosh (10))		$S_{20}$	Ester sulfate	Hexosamine
	Measured as Ba salt	Calculated as Na salt	Measured as Ba salt	Calculated as Na salt			
	per cent	per cent	per cent	per cent		per cent	per cent
Na salt		100		85	2.0	9.5	18
Ba " (from Na salt) . .	67	82	72	89	2.60	9.0	
I, recrystallized . .	44	55	63	77	2.62		
II, recrystallized . .	33	45	66	81	2.71		
III, recrystallized . .	31	38	65	80			
IV, recrystallized . .	25	33	67	82		11.5	
V, recrystallized . .	21	26	60	74	2.87		

This decrease in frictional ratio means that the molecules have changed their shape or their hydration after the treatment with warm acetic acid. Depolymerization of the heparin molecules has not occurred (*cf.* Jorpes), but other changes in the structure of the molecules have obviously taken place.

Qualitative differences also appear in the electrophoresis diagram. In the heparin Roche recrystallized five times, the two components mentioned above were again found, with the mobilities  $u_\alpha = 17.9 \times 10^{-5}$  and  $u_\beta = 15.4 \times 10^{-5}$  close to the values for the initial material. We could calculate from the electrophoresis diagrams that the  $\alpha$  component consti-

tuted 70 per cent and  $\beta$  made up 30 per cent of this preparation, while for the initial heparin Roche the ratio is 1:1.

#### DISCUSSION

The experiments are of particular interest because no agreement is found between different investigators (Charles and Todd (13); Jorpes (1)) as to whether heparin is to be regarded as a homogeneous substance or whether it consists of a mixture of related substances. Kuizenga and Spaulding (14) found that heparin from lung could be fractionated by means of precipitation with acetone into two distinct sodium salt fractions which exhibited different activities. Our experiments show that a similar fractionation of heparin is also possible by various other means. Commercial heparin obviously contains different substances with different properties.

As to the anticoagulant effect of heparin, the ester sulfate component does not play the only rôle. Jorpes (1) had assumed that the decrease in activity following recrystallization was due to depolymerization, basing his opinion on the fact that ester sulfuric acids of other high polymeric carbohydrates exert an anticoagulant effect. This effect was gradually lost when the molecules were depolymerized (15). From our experiments, we can state that the anticoagulant effect of heparin is distinctly more complicated, since no decrease in molecular weight or in ester sulfate content is observable, despite a strong decrease in the anticoagulant effect. As judged from the molecular weight determinations, heparin cannot be reckoned to belong to the very highly polymerized substances, and thus, direct comparisons with the synthetic polysulfuric esters of carbohydrates are not justified. The latter, in spite of their higher polymeric nature, exhibit weaker anticoagulant activity. Therefore, it seems more likely that the amino nitrogen that is set free during recrystallization in warm acetic acid according to Wolfrom *et al.* (8, 9) plays a fundamental rôle in the anticoagulant effect of heparin. With regard to the changes in molecular structure described above, which definitely take place parallel with the liberation of the amino groups, it appears unlikely that amino nitrogen is acetylated, as was previously assumed by Jorpes and Bergström (16).

#### SUMMARY

These investigations show that commercial heparin can be separated by means of electrophoresis and adsorption analysis into fractions with different anticoagulant properties. In the course of repeated recrystallization of heparin in warm acetic acid, according to Wolfrom *et al.*, the inactivation of heparin is not associated with any depolymerization, which was assumed by previous authors.

We express our sincere gratitude to Professor The Svedberg for enabling us to carry out this work. For financial support our thanks are due to Consul General Axel Ax:son Johnson, Stockholm.

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## PANTOTHENIC ACID STUDIES

### IV. PROPIONIC ACID AND $\beta$ -ALANINE UTILIZATION\*

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Wright and Skeggs (1) in a recent publication have investigated the inhibitory effect of propionic acid on the growth of *Escherichia coli*. They observed that this inhibition could be reversed in large measure by small amounts of  $\beta$ -alanine or pantothenic acid, and concluded from their studies that propionic acid operates to prevent the synthesis of  $\beta$ -alanine by *Escherichia coli*.

Although the above hypothesis appears attractive, it occurred to us that at least two other explanations had not been definitely eliminated; i.e., that propionic acid combines with  $\beta$ -alanine, forming an inactive or possibly toxic peptide, or that propionic acid competes with  $\beta$ -alanine for combination within the cell (possibly to a specific enzyme), thus eventually preventing coupling of the pantothenic acid moieties. These possibilities have been investigated in the present paper, and the evidence appears to favor the latter one.

#### EXPERIMENTAL

In order to study the effect of each pantothenic acid moiety upon propionic acid inhibition, two organisms were chosen in which the ability to synthesize the vitamin is more restricted than in *Escherichia coli*. These were *Acetobacter suboxydans*, which requires pantoic acid for growth, and *Saccharomyces cerevisiae*, strain LM (American Type Culture Collection No. 9371), which requires  $\beta$ -alanine.

The testing methods for yeast and acetic acid bacteria were the same as those reported in previous papers (2, 3). The media were adapted to the needs of the present study, so that the effect of propionic and other acids could be observed. Their compositions are shown in Table I.

The sodium propionate solution was prepared by dissolving propionic acid in water and adjusting the pH to that of the medium used. Sodium

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acetate solution was made similarly from the anhydrous salt. In order to avoid volatilization of fatty acids during steaming, the solutions were sterilized by filtration and transferred aseptically to the test flasks or tubes immediately prior to inoculation with organisms.

Propionyl- $\beta$ -alanine was prepared by condensing propionyl chloride with  $\beta$ -alanine in a dilute sodium hydroxide solution. After acidification, the resulting mixture was evaporated *in vacuo* to dryness. The residue was

TABLE I  
Basal Media

	Organism	
	<i>Acetobacter suboxydans</i>	<i>Saccharomyces cerevisiae</i> , strain LM
Glycerol.....	100 gm.	0 gm.
Glucose.....	5 "	20 "
Casein hydrolysate, vitamin-free.....	10 "	0
Norit-treated peptone.....	5 "	0
"    liver concentrate.....	2 "	0
Tryptophan.....	200 mg.	0
Cystine.....	150 "	0
Ammonium sulfate.....	0	3 "
Potassium dihydrogen phosphate.....	0	2 "
Salt Solutions 1 and 2 (4)*.....	0	1 ml. each
"    A and B(5).....	1 ml. each	0
Inositol.....	0	15 mg.
Riboflavin, pyridoxine.....	0	200 $\gamma$ each
Nicotinic acid, <i>p</i> -aminobenzoic acid.....	200 $\gamma$ each	200 " "
Biotin.....	0	1 "
Folic acid.....	0	2 "
Thiamine.....	100 $\gamma$	100 "
Water to.....	1 liter	1 liter
pH.....	6.0	4.8-5.0

\* Bibliographic reference.

exhaustively extracted with anhydrous petroleum ether in a Soxhlet extractor. The propionyl- $\beta$ -alanine was obtained as a light yellow sirup by extraction with dry ethyl ether. Calculated for  $C_8H_{11}O_3N$ , N 9.65 per cent, neutralization equivalent 145.6; found, N (micro-Kjeldahl) 9.50 per cent, neutralization equivalent 149.

Propyl propionate was prepared by refluxing propyl alcohol and propionic acid in the presence of 10 per cent sulfuric acid. The ester was separated, dried, and redistilled three times, and the fraction boiling at 122-124° was collected.

## Results

Table II shows the effects of sodium propionate and sodium acetate on the growth of yeast. Inhibition at low levels of  $\beta$ -alanine was virtually complete in the presence of 1 mg. of propionate (0.0015 M), whereas acetate was much less potent. This is in keeping with Wright and Skeggs' observations with *Escherichia coli* (1). At low levels of  $\beta$ -alanine, the ratio of propionate to  $\beta$ -alanine to produce 50 per cent inhibition was roughly 100:1; for complete inhibition, a ratio of about 10,000:1 was required. The ratio was not constant, since relatively more  $\beta$ -alanine was necessary to overcome increasing doses of propionate, and at higher levels of the latter compound growth could not be completely restored, even by 160  $\gamma$  of  $\beta$ -alanine.

TABLE II

*Growth of Saccharomyces cerevisiae, Strain LM (No. 9371), in Presence of Sodium Propionate and Sodium Acetate*

$\beta$ -Alanine	Sodium propionate								Sodium acetate		
	Molar concentration										
	0	0.0003	0.002	0.004	0.006	0.016	0.032	0.072	0.016	0.032	0.072
$\gamma$	Optical density, 2 - log G										
0.0	0.025		0.010	0.005	0.005	0.015	0.015	0.000	0.025	0.025	0.020
0.2	0.090		0.025	0.025	0.025	0.025	0.015	0.000	0.080	0.070	0.070
0.5	0.220	0.050	0.035	0.030	0.030	0.030	0.025	0.000	0.210	0.220	0.120
1.0	0.360	0.155	0.150	0.075	0.080	0.055	0.030	0.000	0.300	0.260	0.140
2.0	0.390	0.280	0.250	0.165	0.110	0.080	0.030	0.000	0.330	0.330	0.170
5.0	0.430	0.350	0.320	0.270	0.180	0.120	0.045	0.000	0.370		0.230
20.0	0.430	0.440			0.280						
160.0	0.450	0.450			0.300						

Tubes containing 0.5 mg. of glutamic acid gave better growth, but this was considered to be due to the stimulating effect of the amino acid as previously reported (6), and presumably is not directly related to propionate.

The methyl and propyl esters of propionic acid showed feeble inhibition, but propionyl- $\beta$ -alanine stimulated growth slightly. Both effects may be due to hydrolysis by the yeast, or possibly (in the latter case) to contamination with traces of  $\beta$ -alanine. The results are summarized in Table III.

In other experiments (data not given), the reversing power of  $\beta$ -alanine and pantothenic acid in yeast was compared to that of other vitamins in the presence of various levels of propionic acid. An increase of 2- or 6-fold in the concentrations of the vitamins present in the basal medium had no effect on propionate inhibition. 0.5  $\gamma$  of pantothenic acid, on the other hand, completely reversed the effect of 0.25 mg. of propionate (0.00037 M).



The intact vitamin was thus about 40 times as effective as  $\beta$ -alanine in this respect, whereas pantoic acid had no reversing power whatever, even in doses as large as 40 mg. per tube. It would appear, therefore, that the propionate effect is related to  $\beta$ -alanine utilization, even though  $\beta$ -alanine cannot completely overcome high levels of propionate.

TABLE III

*Turbidimetric Response of Yeast Strain LM to Propionic Acid Esters and Propionyl- $\beta$ -alanine in Presence of  $\beta$ -Alanine*

$\beta$ -Alanine	Methyl ester			Propyl ester		Propionyl- $\beta$ -alanine			
	0 mg.	5 mg.	25 mg.	0 mg.	25 mg.	0 $\gamma$	0.1 $\gamma$	10 $\gamma$	100 $\gamma$
$\gamma$	Optical density								
0.0	0.020	0.010	0.005	0.040	0.030	0.040	0.040	0.050	0.490
0.2	0.050	0.050	0.020	0.105	0.040	0.115	0.120	0.130	0.500
2.0	0.260	0.280	0.100	0.410	0.040	0.530	0.500	0.510	0.520
5.0	0.360	0.330	0.200	0.450	0.100	0.520	0.510	0.520	0.520

TABLE IV

*Growth Response of Acetobacter suboxydans to Propionate in Presence of Pantoic Acid and  $\beta$ -Alanine*

Pantoic acid	Na propionate					Propyl propionate	Na propionate (0.048 M) + 5 $\gamma$ pantoic acid	
	0 M	0.012 M	0.024 M	0.048 M	0.096 M	0.048 M	$\beta$ -Alanine	
$\gamma$	Optical density							
0.0	0.030	0.030	0.030	0.035	0.030	0.030	mg.	
0.5	0.430	0.340	0.300	0.150	0.060	0.290	0.1	0.230
1.0	0.620	0.570	0.540	0.200	0.060	0.440	0.2	0.320
5.0	0.630	0.610	0.540	0.240	0.060	0.400	0.5	0.420
15.0				0.230			1.0	0.400
45.0				0.280				

The effects of sodium propionate on *Acetobacter suboxydans* are summarized in Table IV. This organism, like *Escherichia coli* (1), synthesizes  $\beta$ -alanine, and, like the latter organism, requires much higher levels of propionate for inhibition than does yeast. Likewise, relatively large amounts of extra  $\beta$ -alanine are needed to relieve the inhibition. It is again evident that pantoic acid has no effect on the inhibition, as was shown for yeast and for *Escherichia coli* (1).

Adenine, which was found to potentiate propionate inhibition in *Escherichia coli* (1), had no effect upon yeast or *Acetobacter suboxydans*.

## DISCUSSION

Following the development of the Woods-Fildes theory of metabolite-antimetabolite relationships (7, 8), experiments of the type carried out by Harris and Kohn (9) and by Shive and Macow (10) with vitamin analogues as inhibitors have rather clearly established the principle that a growth factor which competitively counteracts a specific inhibitor can be replaced with greater effectiveness by other compounds which normally are formed from the growth factor. This is explained by assuming that the inhibitor retards only the enzyme system in which it competes, and that it is incapable of entering into successive biosynthetic reactions in the cell. Viewed in reverse fashion, this concept has also been utilized (10, 11) to identify precursors of growth factors, against which specific inhibitors may be much more effective than against the growth factors themselves. Since propionate is far more effective against  $\beta$ -alanine than against pantothenic acid, application of the above principle would indicate that propionate interferes with reactions which precede the utilization of the vitamin itself, such as the synthesis of  $\beta$ -alanine or the two reactions proposed at the outset of this paper.

The first alternative mechanism proposed to explain propionate inhibition, namely that  $\beta$ -alanine combines with propionic acid, now seems improbable, however, inasmuch as  $\beta$ -alanine is incapable of completely reversing higher levels of propionate. Moreover, the great superiority of pantothenic acid over  $\beta$ -alanine in reversing the inhibition makes such an explanation seem doubtful, as does the fact that propionate is several hundred times as effective an inhibitor as acetate, and is correspondingly more easily counteracted by  $\beta$ -alanine. The obviously closer structural relationship between propionic acid and  $\beta$ -alanine has been pointed out (1).

The final question of whether propionate inhibits  $\beta$ -alanine utilization directly can be answered by the experiments with yeast. Since this organism cannot synthesize the nutritive, it should be relatively insensitive to propionate if the latter were merely capable of blocking  $\beta$ -alanine synthesis (1). The observed great sensitivity to propionate and the ready reversal by  $\beta$ -alanine, on the other hand, indicate that the utilization of  $\beta$ -alanine is impaired by this inhibitor. Such impairment, it would seem, could be brought about only by (a) either the combination of propionate with pantoic acid or (b) the combination of propionate with some other cellular constituent, thus resulting in the failure of  $\beta$ -alanine to become similarly combined. Since it has already been shown that propionate does not combine with pantoic acid, the last mechanism appears to be the most likely one.

In a previous publication dealing with the effects of various inhibitors related to pantoic acid and pantothenic acid (12), it was suggested that pantoic acid is attached to cellular enzymes through one or both of its

hydroxyl groups. This suggestion is in line with the observation by Lipmann and coworkers (13) that the bulk of the vitamin is in bound form, and that it may be released in large degrees by digestion with a phosphodiesterase. The hydroxyl groups of pantothenic acid appear to be logical points for phosphate attachment.

In an attempt to understand the manner in which  $\beta$ -alanine becomes attached to pantoic acid in these organisms, the growth-promoting activity of  $\beta$ -alanine in low concentrations and the ease with which it reverses propionate inhibition appear important. Both of these observations suggest that this nutriline is also attached to an enzyme, although the competitive behavior of propionate points to a weak attachment. Moreover, the eventual coupling of  $\beta$ -alanine to pantoic acid would require any previously formed  $\beta$ -alanine-protein complex to be easily dissociated. In this scheme, the protein itself would thus serve as a catalyst, bringing about the coupling of the pantothenic acid moieties.

Although the above scheme is speculative, it accounts satisfactorily for all of the recorded observations regarding the formation and utilization of pantothenic acid which have come to our attention. The superiority of pantothenic acid over  $\beta$ -alanine in reversing growth inhibition (not only by propionate but also by glutamic acid and other amino acids (6, 14) is easily explained, if it is assumed that these compete with  $\beta$ -alanine for attachment to the protein, as explained above. Such competition would, of course, be impossible wherever pantothenic acid serves as the growth promoter.

The authors wish to acknowledge the technical assistance of Ruth S. Langdon.

#### SUMMARY

The inhibition of the growth of yeast and *Acetobacter suboxydans* by propionate has been observed, and the relation of  $\beta$ -alanine to this inhibition has been studied.

From the results obtained, it appears that propionate inhibits growth by competing with  $\beta$ -alanine for attachment within the yeast cell, thereby preventing the coupling of the pantothenic acid moieties.

The preparation of propionyl- $\beta$ -alanine is described.

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## THE SYNTHESIS OF BACTERIAL VIRUSES\*

### I. THE SYNTHESIS OF NUCLEIC ACID AND PROTEIN IN *ESCHERICHIA COLI* B INFECTED WITH $T_2^{+}$ BACTERIOPHAGE

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The  $T_2^{+}$  and  $T_4^{+}$  viruses are tadpole-shaped bacteriophages (1, 2) whose biological properties and relations to other bacteriophages have been reviewed by Delbrück (3). The viruses have been isolated by differential centrifugation from bacterial lysates (2, 4, 5) and some chemical and physical properties of the concentrates have been studied (6-8).

About 40 per cent of deoxyribonucleic acid (DNA) has been found in preparations of the viruses (4, 7) and a salt of polymeric DNA has been isolated from active or irradiated  $T_2^{+}$  and  $T_4^{+}$  in high yield (9). Taylor has claimed that ribonucleic acid (RNA) is also present in variable amounts in  $T_2^{+}$  (7). This report has been analyzed elsewhere (9). We have been unable to find evidence for the existence of RNA in preparations of  $T_2^{+}$  and  $T_4^{+}$ ; at least 99 per cent of the P content of the viruses can be accounted for as DNA (4, 5, 9). The data in this and the following study (5) show that cells infected with  $T_2^{+}$  do not appear to synthesize or metabolize RNA, despite a rapid synthesis of DNA in these cells. It appears unlikely, therefore, that RNA is a constituent of either virus.

It has been observed in the *Escherichia coli*- $T_2$  systems that (1) the normal host cells synthesized far more RNA than DNA (7), (2) the virus contained large amounts of DNA and no RNA, and (3) during the multiplication of the virus, bacterial multiplication was inhibited without affecting the rate of  $O_2$  consumption or the R.Q. of the bacteria (4). The following questions were posed: (1) Which, if any, of the nucleic acids was synthesized after infection? (2) What happened to phosphorus and nitrogen assimilation during infection?

#### *Materials and Methods*

In the bacteriophage systems, independent host cells may be simultaneously infected with several virus particles. Large numbers of infected host cells may thereby be studied, all in approximately the same phase of virus

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production. The multiple infection technique provides a method of study of a single generation of virus multiplication.

*The  $r^+$  Factor*—According to Hershey (10) and Doermann (personal communication) these viruses possess the hereditary property termed  $r^+$  which affects lysis as follows: In a dilute suspension of infected cells, essentially maximal liberation occurs in about 30 to 40 minutes. In a concentrated suspension, the first small percentage of infected cells that lyse liberate  $r^+$  virus particles which are rapidly reabsorbed to the other non-lysed cells. The adsorption of  $r^+$  virus to a cell which already has  $r^+$  virus within it inhibits the lysis of the cell for 60 to 90 minutes more, permitting continuing virus multiplication within the lysis-inhibited cell. The phenomenon is of assistance technically, because larger amounts of new substance can accumulate in the extended period of multiplication within the host. The  $r^+$  characteristic may be lost by spontaneous mutation to produce an  $r$  virus which is not lysis-inhibitory.

*Preparation of Virus*—The purification of  $T_2r^+$  from lysates in the ammonium lactate medium (F) and nutrient broth (N) by differential centrifugation has been described (4). Most virus concentrates used in this study, whether derived from F or N, *i.e.*  $T_2$ -F or  $T_2$ -N, were purified by the differential centrifugation procedure from lysates resulting from a single generation of virus production under conditions of multiple infection (5). Variations in the preparation of infecting virus did not appear to affect the course of the phenomena described below. Virus concentrates were suspended in 0.85 per cent NaCl. Their assay has been described (8).

*Preparation of Ultraviolet-Irradiated Virus*—Purified virus concentrates in 0.85 per cent NaCl were irradiated in the apparatus described and kindly loaned by Dr. W. Henle of this Hospital (11). The agitated virus solution was kept 2 inches from the lamp for 20 to 30 second periods to reduce the titer of active virus by a factor of  $10^5$  to  $10^6$ .

*Preparation of Normal and Infected Bacteria*—The growth, assay, and turbidimetric standardization of the bacteria, *Escherichia coli* B, from F medium and broth (N) have been described (8, 12). In aerated F and N at  $37^\circ$ , the division times in the logarithmic phase were 60 and close to 30 minutes, respectively. In most experiments, the concentration of bacteria was about  $2 \times 10^8$  per cc.; in studies involving protein synthesis, bacteria were grown to a concentration of  $2 \times 10^8$  per cc., centrifuged, and resuspended at a concentration of  $10^9$  per cc. At the desired bacterial concentrations, virus was added to produce multiple infection; *i.e.*, three to five virus particles per cell. Infected cultures were vigorously aerated. The experiments were done in F medium unless otherwise stated.

*Analysis*—Aliquots of the cultures were precipitated by the addition of 50 per cent trichloroacetic acid (TCA) to give a final TCA concentration of

5 per cent. The mixture was chilled 15 to 30 minutes and centrifuged 10 minutes at 4000 R.P.M. The supernatant fluids were poured off and the tubes were drained. Two additional washings with cold 5 per cent TCA were used if the end-product was to be analyzed for phosphorus or nitrogen.

Phosphorus was determined by the King procedure (13). Nitrogen was estimated by the micro-Kjeldahl method, with a 1 hour digestion in a  $\text{H}_2\text{SO}_4\text{-K}_2\text{SO}_4$  mixture with  $\text{CuSO}_4$  catalyst. 2 drops of 30 per cent  $\text{H}_2\text{O}_2$  were then added and the digestion was continued for 1 hour.

DNA was determined on the drained sediments by the diphenylamine procedure (14) in Klett colorimeter tubes. With bacterial substance corresponding to  $1$  to  $2 \times 10^9$  bacteria, the heated mixtures gave colorimeter readings with a No. 540 filter of about 20 before infection to  $>100$  near the end of virus synthesis. The values of DNA are given in terms of free acid, since the standard sample of the neutralized DNA was standardized by its P content, the free acid (DNA) being considered to have 10.0 per cent P. The theoretical P content of a polymeric DNA consisting of repeated tetranucleotides is 9.97 per cent.

Pentose was determined by the Mejbaum modification of the Bial reaction (15) on TCA precipitates. The pentose in pyrimidine nucleotides is not estimated by this procedure. The standard for these estimations was D-ribose and the color was read in a Klett-Summerson photoelectric colorimeter with a No. 660 filter. Determinations on unsedimented samples rich in lactate developed green colors slightly modified by brown; nevertheless the relative constancy of these values indicated that pentose, including any non-RNA pentose, did not accumulate. The absence of a change in pentose during synthesis is considered to signify the constancy of RNA. The RNA content of *Escherichia coli* B was about 3 times the DNA content (7).

*Substances*—The preparation of DNA has been described (8). D-Ribose had been prepared by the late Dr. P. A. Levene of the Rockefeller Institute. 5-Methyltryptophan was generously supplied by the Winthrop Chemical Company. Parenamine is an acid hydrolysate of casein, supplemented by tryptophan, and was obtained from Frederick Stearns and Company. It was neutralized before use. L-Glutamic acid was a Merck product.

#### EXPERIMENTAL

*Synthesis of DNA in Infected Cells*—A bacterial culture (200 cc. at  $3.5 \times 10^8$  cells per cc.) was divided into two portions, to one of which was added 0.32 cc. of a  $\text{T}_2$  concentrate with a titer of  $3.1 \times 10^{11}$  per cc. The turbidities and the virus and bacterial contents of the suspensions were determined; DNA was estimated in 8 cc. aliquots. The data on this experiment are presented in Fig. 1.



In the uninfected culture the viable bacterial count, turbidity, and DNA increased exponentially for 3 hours. In other experiments of this type, the slope of the log turbidity-time curve was frequently less than that of the log viable count-time curve, indicating a decrease of the size of individual bacteria during this period. In the infected culture, the count of bacteria capable of forming colonies fell to a very small value as virus was adsorbed. The number of infectious centers fell to the level of infected bacteria, *i.e.* to about one-third of the original virus added, and remained constant for a

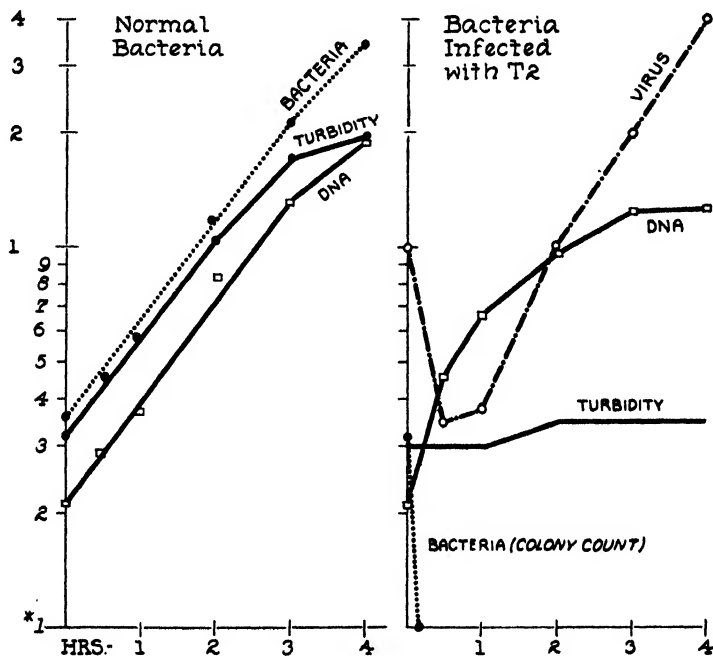


FIG. 1. Desoxyribonucleic acid (DNA) synthesis in normal and T<sub>2</sub>r<sup>+</sup>-infected *Escherichia coli* B in F medium. The ordinate represents  $10^8$  bacteria or virus particles per cc., 0.01 mg. of DNA, or 10 units of turbidity.

period. At 2 hours, virus appeared, becoming maximal at 5 hours (5). Despite the liberation of virus concomitant with the lysis of the host, turbidity remained roughly constant. A possible explanation appears to be that lysis of r<sup>+</sup> virus-infected cells, under the conditions given, begins without considerable fragmentation and, proteolysis being relatively weak, large fragments of cellular debris remain for many hours and scatter light.

In the uninfected culture the turbidity and DNA almost doubled in the 1st hour. In the infected culture, during the same time period, there was no significant increase in turbidity, since only a few per cent of bacteria were

lysed, but the DNA content had more than tripled. The rate of DNA synthesis per infected cell in the 1st hour of the experiment was about 4 times that of normal dividing cells. Therefore, despite the inhibition of bacterial multiplication, the synthesis of this virus constituent was markedly increased. It will be shown in Paper II that much of this newly synthesized DNA can be isolated in the virus (5).

*Effect of 5-Methyltryptophan on Nucleic Acid Synthesis*—5-Methyltryptophan (5-MT) will inhibit  $T_2$  or  $T_4$  multiplication in this system without any effect on  $O_2$  consumption (16). The compound is inhibitory because of its competition with tryptophan at sites of protein synthesis (17). The inhibitory effect of 5-MT on virus synthesis is completely reversible on addition of tryptophan (18).

At low bacterial concentration,  $5 \times 10^{-4}$  M 5-MT in F medium was invariably inhibitory in the absence of added tryptophan. At about  $2 \times 10^8$  bacteria per cc., the effect of 5-MT was more variable. Virus multiplication after infection in the presence of 5-MT was inhibited for only 1 to 3 hours. Furthermore, interruption of virus synthesis after it had begun was not always successful at these concentrations, and, when it occurred, seldom lasted more than  $\frac{1}{2}$  hour. Nevertheless, since 5-MT was a specific antime-tabolite for an essential protein constituent, it was of interest to determine whether the inhibition of protein synthesis affected DNA synthesis. It was found that bacteria infected with  $T_2$  in the presence of 5-MT were incapable of synthesizing protein-bound DNA for several hours. A typical experiment is given in the following section.

*Absence of Synthesis of RNA in Infected Cells*—Both DNA and RNA were followed in infected cells in the presence and absence of 5-MT. 1 minute before addition of virus 10 cc. of  $5 \times 10^{-3}$  M 5-MT in F medium were added to a 100 cc. aliquot of a bacterial culture. After infection, the turbidities were determined as well as the DNA and pentose contents of the TCA precipitates. The results are given in Fig. 2.

In normal cells, RNA was synthesized at a rapid rate (see pentose in Fig. 3). However, throughout infection RNA was constant, in contrast to the stimulated synthesis of DNA. RNA was not synthesized in 5-MT-treated infected cells, even when there was inhibition of both virus multiplication and DNA synthesis.

*Nucleic Acid Synthesis in Cells Treated with Ultraviolet-Inactivated Virus*—Ultraviolet-irradiated virus inhibited bacterial multiplication without change in the rate of  $O_2$  consumption or R.Q., although virus was not being synthesized (4). It was of interest to know whether nucleic acid was synthesized under these conditions. To one of three aliquots of a bacterial culture was added a  $T_2$  concentrate containing  $5.6 \times 10^{10}$  active virus per cc. To another was added the same amount of previously irradiated concen-

# BACTERIAL VIRUSES. I

trate (active virus content of  $10^8$  per cc.). The data on the turbidity, DNA, and total pentose contents of the cultures are presented in Fig. 3. Aliquots of 8 and 2 cc. were used for the DNA and pentose determinations respectively.

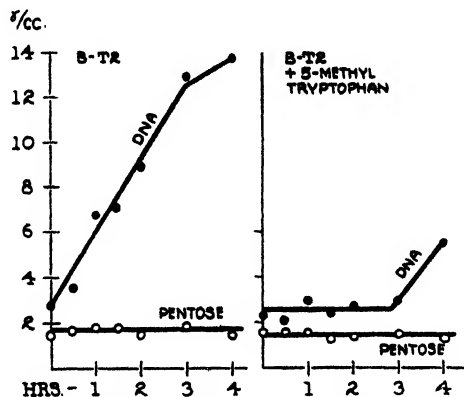


FIG. 2. DNA and pentose synthesis in  $T_{1r}^{+}$ -infected *Escherichia coli* B in F medium in the absence and presence of  $5 \times 10^{-4} M$  5-methyltryptophan.

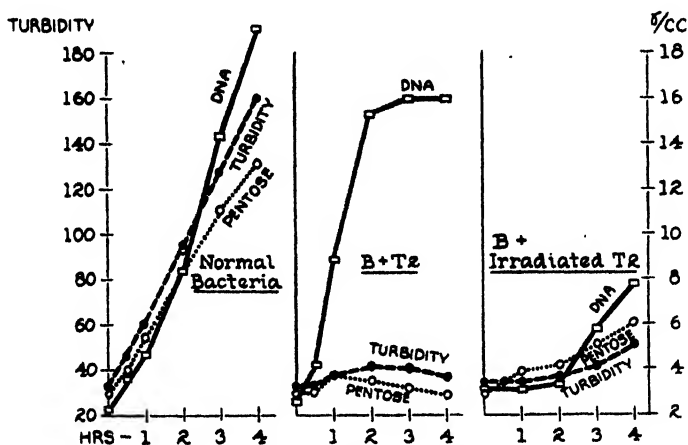


FIG. 3. DNA synthesis in a normal bacterial culture and in bacteria treated with active or irradiated  $T_{1r}^{+}$ .

In the normal bacteria, turbidity, DNA, and RNA increased throughout the experiment. In the bacteria infected with active virus, DNA increased markedly, while pentose remained low. In bacteria treated with irradiated virus, DNA synthesis was completely inhibited over a 2 hour period, during which time total pentose increased slightly. The nature of the late DNA increment in this experiment with irradiated virus is not understood.

**Phosphorus Assimilation and DNA Synthesis**—Phosphate assimilation in the protein-bound phosphate fraction in normal and infected bacteria was examined to see whether phosphorus-containing compounds besides DNA were being synthesized. Infected and normal cultures at  $2 \times 10^8$  bacteria and  $10^9$  T<sub>2</sub> per cc. were incubated for 2 hours. In this interval the turbidity of the infected culture increased only 10 per cent. The DNA, nitrogen, and phosphorus contents of protein-bound constituents are presented in Table I.

In the normal bacteria, one-quarter of the protein-bound phosphorus was DNA-P; the remainder was for the most part RNA-P, but also included small amounts of phospholipides (7). As the bacteria approached the end of their logarithmic phase after 2 hours, a slight decrease was observed in the proportion of phosphorus appearing in DNA. Nevertheless, the molar

TABLE I  
*Synthesis in Normal and T<sub>2</sub>-Infected Escherichia coli B*

Culture	N per 50 cc.	P per 50 cc.	DNA per 50 cc.	DNA-P Total P	Molar ratios, N:P
	mg.	mg.	mg.		
B at 0 hr.	0.333	0.0602	0.154	0.26	12.2
" " 2 hrs.	1.229	0.2219	0.422	0.19	12.2
B <sub>2</sub> - B <sub>0</sub>	0.896	0.1617	0.268	0.17	12.2
B-T <sub>2</sub> at 0 hr.	0.307	0.0685	0.179		
" " 2 hrs.	0.430	0.1053	0.545		
(B-T <sub>2</sub> ) <sub>2</sub> - (B-T <sub>2</sub> ) <sub>0</sub>	0.123	0.0368	0.366	0.99	7.38

N:P ratio was essentially unchanged in the culture throughout this period. Grossly, then, normal bacteria synthesized normal bacterial constituents in nearly the same proportions in which they were originally present.

In infected bacteria, the increments of nitrogen and phosphorus were far less than in the normal dividing bacteria. However, the amount of DNA formed in infected cells was greater than that formed in 2 hours in the normal culture. The DNA in infected cells almost exactly accounted for the phosphorus assimilated by those cells.

In this experiment the N:P ratio of the increment in the infected cells was 7.4, in contrast to 12.2 for normal cells. The former figure is the ratio characteristic of T<sub>2</sub>r<sup>+</sup> which has been found to be 7.2 to 7.6 when isolated from F or N lysates. Thus the material synthesized in infected cells in a 2 hour interval had the N:P and DNA-P:P ratio characteristic of T<sub>2</sub>r<sup>+</sup> bacteriophage.

**Rates of DNA and Protein Synthesis**—It appeared that virus-infected cells synthesized virus or virus components alone, in contrast to normal bacteria which synthesized several types of substances, such as nucleic acid, proteins,

and enzymes. Studies were undertaken to determine whether exponential increase of these constituents or some other types of growth curve characterize virus reproduction in infected cells. The former, as described in Fig. 1, is characteristic of DNA in cells in a normal culture under the conditions employed.

Synthesis was first studied in the normal period of virus synthesis, the non-lysis-inhibited period. In a typical experiment, bacteria were concentrated by centrifugation and resuspended in F medium. After infection with five virus particles per cell aliquots for total N and DNA analyses were removed at 5 minute intervals. The DNA increments were converted to nucleic acid N by multiplying by the factor 0.169. Protein N was considered to be

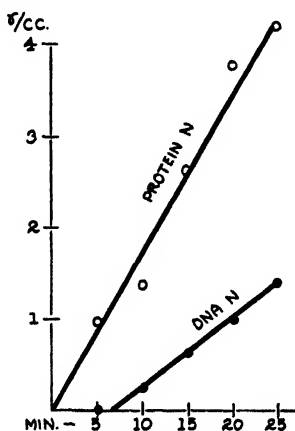


FIG. 4. DNA and protein synthesis in *Escherichia coli* B infected with  $T_2r^+$  virus before the onset of lysis inhibition. The ordinate presents increments over initial values.

total N minus DNA-N. The increments of both protein N and DNA-N are presented in Fig. 4. The following points were observed: (1) The curves of synthesis of DNA and protein in infected cells were linear and not exponential; (2) protein synthesis started at the beginning of infection; (3) the increment in DNA appeared from 7 to 10 minutes after protein synthesis began; (4) if the increment in DNA were converted to an increment in P, the ratio of the rates of increment of N to P in the experiment in Fig. 4 was found to be 12.4, the value for normal bacteria.

The most active concentrate of  $T_2$  studied had  $1.6 \times 10^9$  infectious units per microgram of DNA. In five experiments, DNA increments of 2.2 to 2.6  $\gamma$  were obtained by  $2 \times 10^8$  cells in 25 minutes, yielding an equivalent of seventeen to twenty  $T_2$  particles per cell in that interval. Since our most active  $T_2$  preparation probably contained some inactive virus, this represents a minimal figure. Nevertheless, this agrees with the amount of  $T_2$

liberated from *Escherichia coli* B in F medium; *i.e.*, about twenty virus particles per cell (18).

In four experiments of this type the following ratios of the rates of increment of N to P were obtained: 13.2, 12.6, 11.7, and 12.4. Therefore, in the non-lysis-inhibited system, *i.e.* before 30 minutes, the ratio of the rates of utilization of N and P was approximately the same as in the normal cells. However, in four of five experiments in which these rates were determined over a 2 hour period in infected cells, it was observed that the rate of protein synthesis was sharply reduced between 30 and 50 minutes, while the rate of DNA synthesis was unchanged, as in Fig. 5. This effect was possibly due to lysis inhibition appearing soon after the first liberation of and readsorption of  $r^+$  virus. Thus, after 30 minutes, the ratio of N to P continually de-

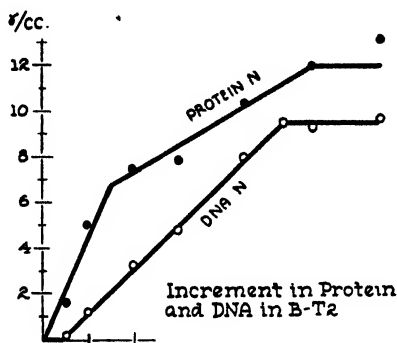


FIG. 5. DNA and protein synthesis in  $T_2 r^+$ -infected *Escherichia coli* B before and after establishment of lysis inhibition.

creased until it approached that of virus. At this point, occurring at about 2 hours, synthesis of DNA abruptly stopped. At this time virus began to appear and a rapid decrease in TCA-precipitable N ensued, indicative of proteolysis accompanying bacteriolysis.

**Inhibition of Protein Synthesis by Irradiated Virus**—Ultraviolet irradiation reduced the activity of  $T_2$  by a factor of  $8.5 \times 10^4$ . Fully active  $T_2$  or irradiated virus was added to bacterial cultures concentrated to  $10^9$  per cc. Aliquots were removed for N and DNA analysis at 0 and 120 minutes. Data on the N and DNA increments after 2 hours are presented in Table II. In the bacteria infected with active  $T_2$ , synthesis of DNA and protein occurred in the ratio approximating that of virus. On the other hand, the bacteria which adsorbed ultraviolet-inactivated virus did not increase their protein-bound N content after a 2 hour interval.

**Initiation of DNA Synthesis**—It has been demonstrated that DNA does not increase for 7 to 10 minutes after infection. Four lines of evidence sug-

gest that this represented the time necessary for the synthesis of virus peptides as a prerequisite for DNA synthesis. (1) Protein was synthesized from the beginning of infection. That this was indeed virus protein was suggested by the viral ratio of N to P at the end of the lysis-inhibited multiplication. (2) DNA synthesis stopped when the excess of protein was markedly depleted as the final ratio approached that of virus. (3) 5-Meth-

TABLE II  
*Inhibition of Protein Synthesis by Irradiated Virus x*

Culture	Time	DNA-P per cc.	Total protein-bound N per cc.	Molar ratio, N:P
	hrs.	$\gamma$	$\gamma$	
B-T <sub>2</sub>	0	1.45	40.2	7.80
"	2	6.00	56.5	
Increment		4.55	16.3	
B-irradiated T <sub>2</sub>	0	1.56	40.4	
"	2	1.96	40.4	
Increment		0.40	0.0	

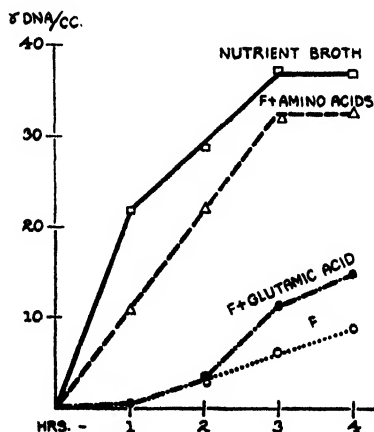


FIG. 6. DNA synthesis in *Escherichia coli* B of the same origin infected in different media by T<sub>2</sub>r<sup>+</sup>. The ordinate presents increments over initial values.

yltryptophan prevented protein synthesis and thereby prevented DNA synthesis. (4) It was possible to reduce the time for the beginning of DNA synthesis and increase the rate of DNA synthesis by increasing the rate of peptide synthesis. This was most clearly demonstrated by the following method. *Escherichia coli* B was grown in broth, instead of in F medium. When this organism was transferred to F medium and washed in F, its synthetic abilities were much slower than F-grown organisms. Under these

conditions, infection in F medium resulted in an extended latent period of virus synthesis and DNA synthesis, with a low yield of virus, as compared to these functions in broth (19).

Bacteria were grown in broth to  $1.5 \times 10^8$  per cc., centrifuged, and washed twice with F medium. The washed bacteria were suspended at their original concentration in broth, F, F + casein hydrolysate (1 mg. per cc.), or in F + glutamic acid at 200  $\gamma$  per cc., respectively, and infected with T<sub>2</sub>. From the DNA analyses presented in Fig. 6, it may be seen that the nutrient broth and amino acid-fortified F medium markedly stimulated DNA synthesis. Some of the difference in the rate of virus and DNA synthesis in these two media can be reduced by further supplementation with purines and pyrimidines (19). Glutamic acid was the most active single amino acid supplement. However, a mixture of amino acids was more efficacious in stimulating virus synthesis and DNA synthesis.

Finally, a complete medium has been developed which supported a rate and amount of virus multiplication closely approximating that in nutrient broth (19). Omission of any one of eight amino acids from the complete defined medium consisting of F, L-amino acids, purines, and pyrimidines resulted in an extended latent period of DNA synthesis. This procedure has been used to detect nutritional requirements for virus multiplication (12).

#### DISCUSSION

After infection, phosphate was apparently channeled into the formation of only that nucleic acid characteristic of virus, namely DNA. The site of the shunt in the pathways of nucleic acid synthesis producing this effect is clearly of utmost importance in understanding the precise nature of the parasitic process in this system. If ribose-5-phosphate was the precursor to both ribose-3-phosphate of RNA and deoxyribose phosphate of DNA, it is conceivable that inhibition of the formation of the ribose-3-phosphate could result in the increased formation of deoxyribose phosphate. Furthermore, since RNA is a common constituent of cellular structures such as mitochondria and microsomes, which are the sites of numerous enzymes, the inhibition of the formation of one element (RNA) of these complex structures may prevent the synthesis of the remaining components.

In infected cells, the rates of synthesis of protein and DNA were constant regardless of the number of virus particles formed within the cell. It would therefore appear that the virus does not contain the enzymes for DNA or protein synthesis and that new enzymes have not been synthesized or, if synthesized, were not being used for virus synthesis. Thus the metabolic equipment which synthesizes the most complex virus components are the enzymes of *Escherichia coli* B, presumably according to the new models



supplied by the infecting virus particles. That this manner of virus synthesis may be general is supported by the fact of the intracellular character of virus multiplication and the data on the composition of various viruses infecting plants, animals, and bacteria, indicating limited structural and enzymatic equipment in these parasites (4, 9).

A constant rate of synthesis in itself may merely indicate some limiting factor in the duplication process and does not eliminate the possibility of a "doubling" process. The limiting factor may be of the character of limitation of substrate, energy supply, or enzyme sites or combination of these. Regardless of the reason for the constant rate, three types of evidence suggest that the curve of the synthesis of protein-bound DNA is indeed a measure of the rate of virus synthesis. (1) Luria and Latarjet have shown that a single hit ultraviolet inactivation curve is obtained for singly infected cells for the first 7 minutes after infection (20). After this time, which coincides closely with the beginning of an increase in DNA, there occurs a slight deformation in the curve which they interpret as "probably an indication that some multiplication has started by this time." (2) The amount of DNA synthesized by infected bacteria in F medium in 25 minutes approximates the amount contained in the virus liberated from the bacteria at about this time. Similar correlations have been found for *Escherichia coli*-T<sub>2</sub> in other media (12). (3) The data of Doermann (personal communication) indicate that, in lysis-inhibited systems of varying latent periods, the burst size is roughly proportional to the length of the latent period. This suggests linear virus synthesis during these periods. His data are consistent with the continued linear synthesis of DNA during lysis inhibition noted above.

The methods described in this paper have strongly suggested that (1) virus is synthesized from the components of the medium assimilated after infection, and (2) RNA is not synthesized in infected cells. Since by the methods described the possible turnover in compounds such as RNA cannot be measured, isotope techniques were employed to confirm these conclusions. Studies with radioactive phosphorus are presented in Paper II (5).

#### SUMMARY

1. The synthesis of nucleic acid and protein has been studied in T<sub>2</sub> bacteriophage-infected *Escherichia coli* B in simple medium, F. Ultraviolet-irradiated T<sub>2</sub> inhibited the synthesis of these components, in contrast to the action of active virus.

2. In infected cells, only the nucleic acid found in T<sub>2</sub>, DNA, was synthesized in contrast to normal cells which synthesized about 3 times as much RNA as DNA. In infected cells, essentially all the phosphorus going to form protein-bound constituents appears in DNA.

3. 5-Methyltryptophan inhibited DNA synthesis in infected cells.

4. In infected cells, protein and DNA were synthesized at a constant rate in the period of normal virus multiplication, prior to the establishment of lysis inhibition. Protein synthesis was apparent from the beginning of infection; DNA began 7 to 10 minutes later. The ratio of the rates of increment of N and P utilization was similar to that of normal cells.

5. The rate of protein synthesis decreased during the period of lysis inhibition in most of the experiments. At the onset of the final lysis, the ratio of the increments in N and P approached that of virus.

6. The length of the period of onset of DNA synthesis was determined by the rate of synthesis of virus peptides.

7. Some aspects of these phenomena have been discussed.

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## THE SYNTHESIS OF BACTERIAL VIRUSES\*

### II. THE ORIGIN OF THE PHOSPHORUS FOUND IN THE DESOXYRIBONUCLEIC ACIDS OF THE T<sub>2</sub> AND T<sub>4</sub> BACTERIOPHAGES

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It has been shown that *Escherichia coli* B infected by T<sub>2</sub> bacteriophage in a synthetic medium, F, synthesizes protein-bound constituents containing phosphorus and nitrogen at a constant rate (1). Evidence was presented that suggests that the virus-infected cell synthesizes virus constituents solely. This was most clearly indicated in the phosphorus metabolism, since the only phosphorylated protein-bound constituent synthesized in the infected cell was that characteristic of virus; i.e., desoxyribonucleic acid (DNA). The ribonucleic acid (RNA) content of infected cells did not increase.

Although the data presented suggested that the materials formed during infection were derived from the medium as in normal cells, it had not been proved that the nucleic acid phosphorus found in virus was not derived from cellular substance existing prior to infection, or had not been in normal host constituents during infection prior to appearance in virus. It seemed unlikely that the virus nucleic acid was derived from the host DNA, since several times more DNA was synthesized after infection than was originally present. However, it was possible that the RNA of the infected cell possessed an active turnover yielding its phosphorus to DNA nucleotides, while the RNA was continually replaced with nucleotides whose phosphorus was derived from the medium.

Two basic problems were therefore posed. (1) Was the DNA-P of virus derived from the phosphorus in the host prior to infection? (2) Was RNA a precursor of DNA? These hypotheses were tested by means of radioactive phosphorus, P<sup>32</sup>.

#### EXPERIMENTAL

*Conditions for Optimal Virus Yield*—A single generation of virus multiplication was studied under conditions of multiple infection. The possibility was therefore eliminated that the generations of virus produced after the first generation in a medium of known composition would be affected by the

\* The work described in this paper was aided by the Office of Naval Research.

appreciable amounts of complex products, radioactive and otherwise, liberated by lysed cells. Some of the chemical phenomena and basic biological aspects of this system have been described in Paper I (1).

The yield of virus from infected cells under conditions of multiple infection in the aerated F medium at 37° was studied at concentrations of  $5 \times 10^7$ ,  $10^8$ ,  $2 \times 10^8$ , and  $5 \times 10^8$  bacteria per cc. It was found that with both  $T_2$  and  $T_4$  maximal titers were obtained with  $2 \times 10^8$  bacteria per cc. in about 4 to 6 hours. After this time, titers generally decreased markedly, and to a greater extent in  $T_2$  lysates than in  $T_4$  lysates. This was due probably to a combination of several factors, reabsorption to cellular debris, spontaneous thermal inactivation, surface denaturation, etc. In view of this marked loss of titer, it was considered desirable to prepare virus lysates by a single generation in infected cells for a 6 hour period, to minimize the destruction of virus activity.

*Distribution of DNA and Protein-Bound P in  $T_2$  and  $T_4$  Lysates*—Two types of experiments were done. (1) Bacteria were grown in media containing radioactive P, washed several times, and infected in media containing non-radioactive P; (2) bacteria were grown in media free of  $P^{32}$  and infected in the presence of  $P^{32}$ .

The following typical control experiments were performed to indicate whether (1) the experimental conditions employed would permit the isolation of sufficient virus for analytical purposes, (2) virus isolated by these procedures would be very low in inorganic P and otherwise possess the proper chemical properties, (3) the newly synthesized DNA appeared in the virus fraction. Bacteria were grown to  $2 \times 10^8$  per cc. in F medium. Two 125 cc. aliquots were sedimented and the bacteria washed twice with 0.85 per cent NaCl. Each aliquot was resuspended in 125 cc. portions of F medium to which were added an adsorption cofactor, 12 cc. of  $5 \times 10^{-3}$  M tryptophan in F (2). To these bacterial suspensions (A and C) were added small volumes of purified concentrates of  $T_4r^+$ -F and  $T_2r^+$ -F to give cultures  $T_4A$  and  $T_2C$  containing  $6 \times 10^8$  virus particles per cc. The virus concentrates were also added to unwashed bacterial cultures (B and D) in F medium at the same concentrations to yield  $T_4B$  and  $T_2D$ ; B contained  $5 \times 10^{-4}$  M tryptophan. The four infected cultures were assayed periodically; the titers followed the course described previously.

After 5 hours at 37°, the  $T_2$  and  $T_4$  lysates were stored for 13 hours at 4°. Very little inactivation occurred; the final titers were  $T_4A$   $2.0 \times 10^{10}$ ,  $T_4B$   $4.0 \times 10^{10}$ ,  $T_2C$   $1.5 \times 10^{10}$ ,  $T_2D$   $1.5 \times 10^{10}$  active virus particles per cc. The lysates were analyzed for total protein-bound P and DNA (1). They were sedimented at 4000 R.P.M. for 30 minutes and the sediments were washed twice with cold 0.85 per cent NaCl. The supernatant fluids were sedimented at 10,000 R.P.M. for 2 hours, and the supernatant fluids from

this high speed centrifugation, containing more than 5 per cent of the activity of the lysate, were analyzed for protein-bound P and DNA. Virus was isolated from the high speed sediments as described previously (3, 4). The distribution of these substances in the four lysates is given in Table I.

It may be seen that the largest amounts of DNA and P appear in the virus-containing fraction; *i.e.*, the high speed sediment. When this fraction was resuspended in 5 cc. of 0.85 per cent NaCl and centrifuged at 4000 R.P.M. for 30 minutes, approximately 25 per cent of the DNA of the fraction had become insoluble. After dialysis of this supernatant fluid against running water overnight, the once sedimented virus concentrate had DNA-P to total P ratios which were very close to 1.0. These values for isolated dialyzed virus after one differential centrifugation cycle were

TABLE I  
*Distribution of Protein-Bound P and DNA in Lysates of Infected Bacteria*

Virus lysate	Analyses	Initial total	Total lysate	Low speed sediment	High speed supernatant fluid	High speed sediment containing virus
		mg.	mg.	per cent	per cent	per cent
T <sub>4</sub> A, 97 cc.	P		0.210	31	29	40
	DNA	0.213	1.49	25	37	38
T <sub>4</sub> B, 105 "	P	0.141	0.263	19	21	60
	DNA	0.220	1.80	17	21	62
T <sub>2</sub> C, 96 "	P	0.161	0.275	27	22	51
	DNA	0.275	1.63	29	20	51
T <sub>2</sub> D, 92 "	P	0.148	0.260	33	17	50
	DNA	0.221	1.36	35	16	49

T<sub>4</sub>A 1.05, T<sub>4</sub>B 1.00, T<sub>2</sub>D 1.02. Thus the conditions described above yielded fractions with the characteristics of virus whose inorganic P contents were so low as to be undetectable.

Infected cells may synthesize as much as 7 to 8 times as much DNA as was originally present. In many cases, at least 3 times as much DNA was isolated in the virus as was present in the bacteria at the onset of infection. It is considered probable that DNA formed after infection and not recovered in isolated virus was nevertheless originally part of the virus and was lost either by adsorption to debris or by becoming insoluble as a result of high speed centrifugation. No indication has been obtained in preparations of either T<sub>2</sub>r<sup>+</sup> or T<sub>4</sub>r<sup>+</sup> bacteriophage from F lysates that a phosphorylated compound other than DNA is present in either virus. Furthermore, in contrast to the observations of Taylor (5), preparations of these viruses from nutrient broth lysates prepared from infected cells as described above also possessed DNA-P to total P ratios of 1.0 (6). Hence no evidence has

been obtained to confirm the report of Taylor that  $T_2r^+$  virus contains ribonucleic acid (5). It is considered possible that the method of isolating virus from 5 to 6 hour lysates described above assists in the removal in the low speed sediment of bacterial components containing RNA. Since proteolysis in  $r^+$  systems is relatively weak, the prolonged period used by Hook *et al.* (7) in the preparation of lysates may have assisted the degradation of bacterial debris to a size which would not sediment at a low speed but would at high speeds. Proteolysis in  $r^+$  systems in F medium seems even less pronounced than in broth.

*Synthesis of Virus in Labeled Host Cells*—Small inocula of bacteria were grown to  $2 \times 10^8$  per cc. in 125 aliquots of F medium containing 0.02 to 0.05 millicurie of  $P^{32}$  in 13.7 mg. of inorganic phosphate. More than 99 per cent

TABLE II

*Radioactivity of Virus Isolated after Synthesis in  $P^{32}$ -Labeled Cells in Media Free of  $P^{32}$*

isolated	DNA-P Total P in virus	Bacteria	Virus	Relative radioac- tivity, virus P host P
		counts per 10 $\gamma$ P per min.	counts per 10 $\gamma$ P per min.	per cent
$r^{++}$	1.00	222	33	14
$r^{++}\dagger$	1.00	293	53.5	18
$r^{++}$	1.01	37.8	5.9	16
$r^{++}\dagger$	1.06	293	53.3	18

\* Virus to cell ratio 3.0.

† Virus to cell ratio 5.0.

of the P of the bacteria was derived from the P of the medium. The cells were washed twice with 0.85 per cent NaCl. The second washing contained less than 0.1 per cent of the radioactivity of the original medium, as determined on a Geiger-Müller counter, kindly loaned by Dr. H. D. Brunner of the Department of Pharmacology of the University of Pennsylvania.

The radioactive cells were resuspended in 131 cc. of F medium containing  $2.5 \times 10^{-4}$  M tryptophan. Purified  $T_2r^+$  or  $T_4r^+$  virus was added and the infected cultures were incubated for 5 hours and regularly assayed for virus during this interval. The lysates were stored at 4° overnight and virus was isolated as previously described.

The DNA contents of the initial cultures, final lysates, and isolated virus were determined. The radioactivity of the virus was compared with the radioactivity of the uninfected host cell per unit weight of P. Samples of known P content were digested in 1.2 cc. of 60 per cent perchloric acid and diluted to 10 cc. after the solutions were neutralized to pH 4. The counting

chamber was filled by perfusion and held identical aliquots of slightly more than 3 cc. The background was determined between samples and yielded 8 to 11 counts per minute. This was subtracted from the count on the sample. Samples and the time of counting were adjusted to yield 100 to 1000 counts. Comparisons of samples were made at similar total counts.

In Table II are presented data on four experiments of this type, in which the increments in DNA in the infected bacteria and the yields of isolated virus were comparable to the data presented previously. The radioactivity of the virus isolated after synthesis under these conditions was far lower than the radioactivity of the host P. It has therefore been concluded that most of the P organized into virus is derived from the medium after infection.

TABLE III

*Radioactivity of Virus Synthesized in Unlabeled Cells in Media Containing P<sup>32</sup>*

* Virus isolated	DNA-P Total P in virus	F medium	Virus	Relative radio activity, virus P medium P
		counts per 10 $\gamma$ P per min.	counts per 10 $\gamma$ P per min.	per cent
T <sub>2</sub> r <sup>++</sup>	1.04	443	315	71
T <sub>4</sub> r <sup>++</sup>	1.00	840	633	75
T <sub>4</sub> r <sup>+</sup>		422	291	69

\* Virus to cell ratio 5.0.

† Virus to cell ratio 3.0.

*Synthesis of Virus in Unlabeled Host Cells in Media Containing Radioactive Phosphate*—Bacteria were grown in the absence of radioactive P. Virus was added immediately after the addition of 0.02 to 0.05 millicurie of inorganic phosphate and tryptophan for the adsorption of T<sub>4</sub>. The cultures were incubated, and the DNA increments, rates of virus liberation, and isolated virus were studied as described previously.

In Table III are presented some data on experiments of this type. The radioactivity of the virus isolated after synthesis under these conditions was 70 to 75 per cent of that of the inorganic phosphate of the medium. These data confirm the previous conclusion that most of the P organized into virus is derived from the medium after infection.

*Nucleic Acid Turnover after Infection*—Since the P<sup>32</sup> added to the F medium was assimilated in DNA after infection, the distribution of the radioactivity in the infected cell was studied. In a typical experiment bacteria were grown to  $2 \times 10^8$  per cc. in 1 liter of F medium. The cells were centrifuged and resuspended in 100 cc. of F medium. To the culture were added



4.5 cc. of  $T_2r^+$  at  $2.2 \times 10^{11}$  per cc. and 1.5 mg. of P in inorganic phosphate containing about 0.05 millicurie of  $P^{32}$ . The infected culture was aerated vigorously at  $37^\circ$  for 1 hour. At zero time and at 1 hour, aliquots were analyzed for protein-bound P, DNA, and radioactivity.

The counts per minute of the medium were 93 per microgram of P. In 1 hour, the bacterial protein-bound P increased from 0.0109 mg. per cc. to 0.0163 or 5.4  $\gamma$  per cc. Newly assimilated P had a radioactivity of 98 counts per microgram. The DNA had increased from 24 to 72  $\gamma$  per cc., or the ratio of newly formed DNA to original DNA was 2:1.

Following the removal of aliquots after 1 hour, the entire culture was precipitated with 5 per cent trichloroacetic acid (TCA) and the sediment was washed twice in the centrifuge with TCA. The supernatant fluids were pooled. The sediment was washed twice with 95 per cent alcohol and twice with ether. These supernatant fluids were combined with lipid extracts prepared by three extractions of this sediment with boiling alcohol-ether (1:1). The pooled lipid extracts were taken to dryness *in vacuo* and dissolved in 10 cc. of  $CHCl_3$ .

The dried lipid-free sediment was fractionated according to Schmidt and Thannhauser (8). It was incubated with 2 cc. of  $N$  KOH at  $37^\circ$  for 20 hours and chilled. To the solution containing a very slight flocculent precipitate were added 0.4 cc. of 6  $N$  HCl and 2 cc. of 5 per cent TCA. After 30 minutes at  $0^\circ$ , the sediment, containing DNA, was removed by centrifugation and washed with several cc. of water. The DNA fraction was dissolved in 0.1  $N$  NaOH. The combined supernatant fluids were precipitated according to Delory (9); essentially no P was found in this sediment and this fraction was discarded. The supernatant fluid contained ribose-3-phosphate nucleotides and comprised the RNA fraction.

Both the DNA and RNA fractions prepared above were analyzed for DNA. The former contained 6.9 mg. and the latter 0.6 mg. of DNA, or a total of 7.5 mg. of DNA was recovered. The total DNA content of the culture was 7.4 mg. Radioactivity measurements of the RNA fraction were corrected for the radioactivity of the DNA it contained. This comprised 10 per cent of the P of this fraction. The radioactivities of the nucleic acid fractions are summarized in Table IV.

One-half of the P in the RNA fraction consisted of ribose-3-phosphate nucleotides reactive in the Bial reaction (10). Pyrimidine nucleotides derived from RNA and comprising one-half of the total P would not be expected to react. Thus the RNA fraction with the exception of a small amount of DNA contained ribose nucleotides of the expected characteristics.

It may be seen from Table IV that the newly synthesized DNA contained P of essentially similar radioactivity to that in the medium, while the RNA

fraction contained essentially no radioactive P. It may be concluded from this experiment and others which yielded the same result that not more than 2 per cent of the RNA could have been undergoing synthesis involving P of the medium. It is highly improbable that the turnover of this much RNA, if any synthesis actually occurred, could have produced the synthesis of the DNA found. Therefore it appears that RNA nucleotides are not precursors to DNA synthesis in this system.

Since special precautions were not taken to purify the nucleotides of the RNA fraction before estimating the radioactivity, it is possible that even the low radioactivity found (2 per cent) was due to some contaminant, and was not truly significant. In this experiment the lipid P was found to have a radioactivity of about 26 per cent of the P of the medium. Reprecipitation of phospholipide in the presence of carrier sphingomyelin derived

TABLE IV

*Inclusion of Radioactive P into Nucleic Acids of Escherichia coli B Infected with T<sub>2</sub>r<sup>+</sup> Bacteriophage*

Nucleic acid	Total Protein-bound P	Lipide-free bacterial residue	Counts per $\gamma$ P per min.	Corrected counts per $\gamma$ P per min.
	<i>per cent</i>	<i>per cent</i>		
DNA	48	13.8	60	90
RNA	36.5	10.5	8.8	2

DNA, counts corrected for presence of original bacterial DNA; RNA, counts corrected for presence of traces of DNA in RNA fraction.

from beef lung readily reduced the radioactivity sufficiently to suggest the inactivity of the phospholipide fraction.

*Fractionation of Non-Protein P*—It was not found possible to isolate organic phosphate from the TCA supernatant fluids in this experiment, owing to the large excess of inorganic P of the medium. In another experiment, the bacteria were first sedimented to remove the inorganic P of the medium before fractionation. 10 cc. of TCA extract contained 66  $\gamma$  of organic P and 183  $\gamma$  of inorganic P. To this solution, adjusted to pH 8.3 with  $\text{NH}_4\text{OH}$ , were added 1 cc. of 2.5 per cent  $\text{CaCl}_2$  and 2 cc. of a 0.5 per cent suspension of  $\text{MgCO}_3$ . After being chilled, the mixture was centrifuged and the sediment was discarded. The supernatant fluid was precipitated with 4 volumes of alcohol and stored overnight in the ice box. The alcohol precipitate was placed in 3 cc. of water. It contained 44  $\gamma$  of organic P, of which ribose-5-phosphate in some form comprised 75 per cent of the total organic P of the fraction (10). The water-soluble fraction of the alcohol precipitate contained 16.5  $\gamma$  of pentose P in 25  $\gamma$  of organic P. The radioactivity per microgram of P of this fraction was 37 per cent of that of the inorganic

P of the medium. It is not known in what compounds the radioactivity resided.

#### DISCUSSION

It may be seen from the data presented in this paper that the hypotheses suggested in Paper I have been verified by means of the isotope technique. These are (1) that phosphorylated virus constituents are synthesized in the main from P assimilated from the medium after infection, and (2) that RNA after infection has a very low, if any, turnover rate, and is not a precursor of virus DNA.

Nevertheless the radioactivity of the virus P was significantly different from that of the P of the medium. It has been observed that a very small amount of virus multiplication may occur in the absence of external P (11). Therefore it appears that some of the P of the host may be incorporated into virus. It is considered likely that this small amount of P is derived from the intracellular pool of inorganic P or low molecular weight organic P which can equilibrate with P assimilated after infection. This intracellular metabolic pool would be used for the synthesis of DNA after infection and possibly even before the P assimilated after infection. Thus in short term experiments involving r strains the percentage of host P appearing in virus may conceivably be greater than that observed in these long term experiments with r<sup>+</sup> type virus. This remains to be tested.

Many workers have found that protein and DNA syntheses are accompanied by a vigorous RNA metabolism in a wide variety of tissues. In addition it has been reported that there is apparently a conversion of ribose nucleotides to deoxyribonucleotides in the early stages of cleavage of the fertilized sea-urchin egg (12). These data are ably reviewed by Brachet (13). In the system described in these papers, none of these phenomena are to be observed. It appears possible to study the precursors of DNA in this system uncomplicated by the metabolism of RNA.

I am indebted to Dr. Samuel Gurin of this University and Dr. M. Kamen of Washington University for their advice and assistance in the course of these studies. I wish to thank Miss Catherine Fowler for her technical assistance.

#### SUMMARY

The preparation and properties of bacterial lysates after multiple infection have been described. As a result certain isotope experiments requiring viral isolation could be performed. The distribution of the bacterial and viral components in these lysates has been studied. It has been found possible to recover in a purified concentrate of virus much of the P assimilated

and the DNA synthesized after infection. The newly synthesized DNA greatly exceeded the starting DNA of the bacterial culture.

With  $P^{32}$  in the labeled host or labeled medium, the DNA of the virus has been found to be built in the main from the inorganic P of the medium. Ribonucleic acid was not a precursor in the synthesis of this DNA and was essentially inert in infected cells.

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# A SPECTROPHOTOMETRIC STUDY OF BLOOD GROUP A-SPECIFIC SUBSTANCE ISOLATED FROM HOG GASTRIC MUCOSA

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The isolation from hog gastric mucosa of homogeneous preparations of the polysaccharide-amino acid complex possessing the ability to inhibit the isoagglutination of human blood group A cells (1) has been hindered not only by difficulties of manipulation and the presence in gastric mucosa of substances having properties similar to those of blood group A-specific substance, but also by the fact that direct tests for determining the homogeneity of the product are often insensitive and unreliable. In the past the efficacy of procedures used for the isolation and purification of group A substance have been evaluated by serological tests and in a few instances by ultracentrifugal analysis (2), electrophoretic analysis (3), total nitrogen content (3), apparent N-acetyl-D-glucosamine content (4), optical rotation (3), and viscosity (3). Since we had at hand a large number of preparations of group A substance obtained from hog gastric mucosa by a variety of methods (5), it was thought advisable to determine to what extent ultraviolet spectrophotometry could be used for the evaluation of these preparations. In addition such a study would also serve to indicate the importance of the aromatic amino acids as structural elements of group A substance.

The ultraviolet absorption spectra of group O, A, and B substances from human urine and of group A substance from hog gastric mucosa, isolated by Freudenberg (6), were first studied by Gróh, Szélyes, and Weltner (7) and later in greater detail by Weltner (8). These investigators reported that in neutral aqueous solution all of the blood group-specific substances showed weak selective absorption in the region 270 to 290 m $\mu$ , and their results indicated that the purification of group A substance, obtained from hog gastric mucosa, caused the extinction to increase throughout the ultraviolet region, since it was reported that a "purified" preparation and a "highly purified" preparation showed extinction coefficients of  $E_{1\%}^{1\text{cm}}$  at 280 m $\mu$  of 1.8 and 2.8, respectively. We have been

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† Contribution No. 1171.

unable to confirm these results and in fact have observed that preparations of group A substance with greatly enhanced potencies, when compared with starting materials and intermediates, show little absorption in the ultra-violet. However, ultraviolet spectrophotometry is particularly useful for the detection of certain impurities that may be present in preparations of the blood group-specific substances.

#### EXPERIMENTAL

*Absorption Spectra*—Optical densities for 1 cm. of solution at 25° were determined with a Beckman model DU spectrophotometer equipped with quartz cells at every 3 m $\mu$  from 220 to 270 m $\mu$ , every 5 m $\mu$  from 270 to 300 m $\mu$ , and every 10 m $\mu$  from 300 to 350 m $\mu$ . Additional observations were made when the maxima were displaced from the usual 260 m $\mu$  region. The spectra for all solutions were corrected for absorption due to solvent (citrate-phosphate buffer), which was important below 240 m $\mu$ . Extinction coefficients,  $E_{1\text{ cm.}}^{1\%}$ , were calculated from the usual relation,  $E_{1\text{ cm.}}^{1\%} = (\log_{10} I_0/I)/cd$ , where  $d$  is the cell thickness and  $c$  the concentration of solute in gm. per 100 ml. of solution.

*Group A Substance Solutions*—Those group A substance preparations which were used and not described previously (4, 5) are described in Table I. The solutions were prepared by dissolving the preparations in distilled water, taking 10 ml. aliquots, and adding 1 ml. of McIlvaine's citrate-phosphate buffer, pH 7.0. The pH of the resulting mixtures was  $7.20 \pm 0.05$  and the concentration of group A substance 300 to 380  $\gamma$  per ml. In a few instances, where indicated, solutions were prepared similarly at pH  $6.30 \pm 0.05$  and pH  $8.05 \pm 0.05$  by means of McIlvaine's buffers. The final citric acid and disodium phosphate concentrations at pH 6.3, 7.2, and 8.0 were, respectively, 0.0034 M  $\text{H}_2\text{C}_6\text{H}_5\text{O}_7$ -0.0115 M  $\text{Na}_2\text{HPO}_4$ , 0.0016 M  $\text{H}_2\text{C}_6\text{H}_5\text{O}_7$ -0.015 M  $\text{Na}_2\text{HPO}_4$ , and 0.004 M  $\text{H}_2\text{C}_6\text{H}_5\text{O}_7$ -0.0176 M  $\text{Na}_2\text{HPO}_4$ .

*Serological Tests*—The inhibition of the isoagglutination test (4) and the inhibition of the hemolysis test (5) have been described previously. The activities of many of the preparations used in this study were reported in the above papers.

#### RESULTS AND DISCUSSION

Group A substance preparations dissolved in distilled water generally give clear or slightly opalescent solutions. These solutions are colorless when prepared from undegraded preparations and faintly yellow when degraded preparations are used. The solutions show deviations from Beer's law, since the extinction coefficients at a given wave-length and pH increase upon dilution.<sup>1</sup> The extinction values also vary with pH and

<sup>1</sup> Unpublished experiments.

generally increase in the region 220 to 350  $m\mu$  with decreasing pH.<sup>2</sup> The extinction curves of solutions of pH 6 to 8 are not altered upon standing and the observed stability of solutions in this pH range is in agreement with observations made elsewhere by independent methods (3, 9). The increase in absorption of alkaline solutions of group A substance observed by Weltner (8) is probably due to the lability of group A substance under

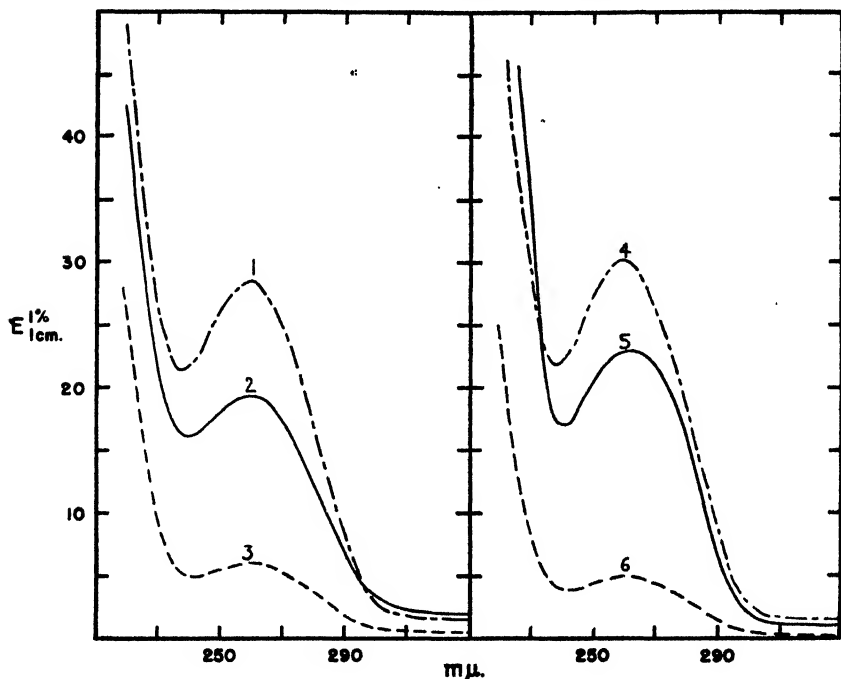


FIG. 1. Extinction curves of fractions isolated from hog gastric mucin by fractionation with ethanol. Curve 1, Fraction C-123; Curve 2, Fraction C-125; Curve 3, Fraction C-128; Curve 4, Fraction C-142; Curve 5, Fraction C-135; Curve 6, Fraction C-143.

such conditions (3). In view of the dependence of absorption upon pH and group A substance concentration, the spectra of all preparations were measured at pH 7.2 and at similar concentrations of group A substance. Typical extinction curves are presented in Figs. 1 to 6, and in Table I the serological properties and the extinction values at 260 and 350  $m\mu$  of many preparations are summarized.

*Extinction Curves of Preparations from Hog Gastric Mucin—Commercial*

<sup>2</sup> The change in extinction values with pH may have a contributing factor due to the difference in salt concentration in the various buffer solutions. Other results indicate that ionic strength also influences spectra.



TABLE I

*Spectral and Serological Properties of Fractions Isolated from Hog Gastric Mucin, Pepsin, and Individual Hog Stomach Linings*

Procedure	Fraction No.	$E_{1\%}^{1\text{cm.}}$		Inhibition of isoagglutination titer, microliters group B serum neutralized per microgram substance	Inhibition of hemolysis titer, micrograms substance inhibiting 50 per cent hemolysis
		260 $m\mu$	350 $m\mu$		
Aqueous suspension of mucin fractionated with ethanol after centrifuging (Sharples) twice at pH 4.4	C-125	19.4	1.7	45 $\pm$ 15	0.18 $\pm$ 0.01
	C-123	28.5	1.5	40 $\pm$ 15	0.24 $\pm$ 0.01
	C-128	5.9	0.5	75 $\pm$ 20	0.11 $\pm$ 0.00
	C-135	22.8	0.9	45 $\pm$ 15	0.18 $\pm$ 0.02
	C-142	30.1	1.3	45 $\pm$ 15	0.24 $\pm$ 0.00
	C-143	4.9	0.3	85 $\pm$ 20	0.11 $\pm$ 0.01
Alcohol fractionation of mucin, procedure of Landsteiner and Harte (2)	R4-F2	30.4	2.2	30 $\pm$ 10	0.12 $\pm$ 0.01
	R4-F3	6.0	1.0	80 $\pm$ 20	
	R4-F4	31.2	1.3	0.1 $\pm$ 0.05	
	R5-F2	41.9	3.2	20 $\pm$ 10	0.11 $\pm$ 0.01
	R5-F3	8.0	0.8	85 $\pm$ 20	
Alcohol fractionation of centrifuged 90% phenol solution of mucin (3) by electrodialysis and alcohol fractionation	C-52	11.6	1.0	50 $\pm$ 15	0.13 $\pm$ 0.01
	C-59	1.7	0.5	90 $\pm$ 30	
	C-61	2.2	0.6	50 $\pm$ 15	
Sodium sulfate fractionation of aqueous centrifuged suspension of mucin (3), followed by electrodialysis	R2-F2A	1.3	0.1	45 $\pm$ 15	0.11 $\pm$ 0.01
	R2-F2B	2.1	0.3	50 $\pm$ 15	
	R2-F2C	3.8	0.9	50 $\pm$ 15	
Electrodialysis of product of alcohol fractionation of mucin, Fraction R5-F3	R8-F1	1.4	0.2	60 $\pm$ 20	
	R8-F2B	7.6	2.1	50 $\pm$ 15	
Treatment of products of alcohol fractionation of mucin with ion exchange resins	C-110	13.2	0.5		0.20 $\pm$ 0.02
	C-165	11.8	0.6		0.20 $\pm$ 0.02
	C-167	3.6	0.5		0.21 $\pm$ 0.02
	C-168	3.4	0.5		0.070 $\pm$ 0.005
Treatment of product of alcohol fractionation of mucin with papain-HCN (2)	R6-F5A	0.6	0.1	25 $\pm$ 10	0.10 $\pm$ 0.01
Treatment of product of alcohol fractionation of mucin with formamide at 150° (2)	R7-F5A	1.7	0.4	10 $\pm$ 5	0.05 $\pm$ 0.005
Alkaline digestion of mucin, followed by selective adsorption and precipitation (18)	C-47	0.8	0.2	30 $\pm$ 10	0.065 $\pm$ 0.01

TABLE I—*Concluded*

Procedure	Fraction No.	E <sup>1</sup> % 1cm.		Inhibition of isoagglutination titer, microliters group B serum neutralized per microgram substance	Inhibition of hemolysis titer, micrograms substance inhibiting 50 per cent hemolysis
		260 m $\mu$	350 m $\mu$		
Alcohol fractionation of Wilson pepsin (19)	C-22	5.0	0.3	70 $\pm$ 15	0.050 $\pm$ 0.005
Autolysis of Wilson pep- sin, followed by alcohol fractionation (2)	C-26	3.7	0.4	35 $\pm$ 10	0.040 $\pm$ 0.005
Peptic digestion of indi- vidual hog stomach lin- ings, followed by 2 alco- hol precipitations (21)*	H1-F3	3.6	0.3	<0.06	>175
	H2-F1	4.7	0.3	90 $\pm$ 30	0.080 $\pm$ 0.005
	H2-F3	2.9	0.2	0.5 $\pm$ 0.2	0.60 $\pm$ 0.05
	H3-F1	7.3	0.3	75 $\pm$ 20	0.13 $\pm$ 0.01
	H3-F2	4.4	0.2	2 $\pm$ 1	0.70 $\pm$ 0.05
	H4-F1	9.6	0.3	<0.06	>175

\* Fraction C-52, material insoluble in 10 per cent (by volume) ethanol; dialyzed. Fraction C-59, soluble in 47 per cent (by volume) ethanol and insoluble in 62 per cent (by volume) ethanol on fractionation of clear supernatant obtained on electro dialysis of Fraction C-52; dialyzed. Fraction C-61, insoluble in 47 per cent (by volume) ethanol on fractionation of clear supernatant obtained on electro dialysis of Fraction C-52. Fraction R2-F2A, insoluble in 30 per cent sodium sulfate; precipitate electro dialyzed; material from supernatant. Fraction R2-F2B, aqueous washings of Fraction R2-F2C. Fraction R2-F2C precipitate obtained on electro dialysis of 30 per cent sodium sulfate precipitate. Fraction C-110, material soluble in 30 per cent (by volume) ethanol but insoluble in 65 per cent (by volume) ethanol from centrifuged mucin suspension. Upon reprecipitation insoluble in 45 per cent (by volume) ethanol; electro dialyzed. Fraction C-165, Fraction C-110 treated twice with alkali-washed Amberlite IR-4 resin. Fraction C-167, Fraction C-110 treated twice with alkali-washed De-Acidite resin. Fraction C-168, material soluble in 45 per cent (by volume) ethanol and insoluble in 65 per cent (by volume) ethanol from centrifuged suspension, dialyzed, and then treated twice with alkali-washed De-Acidite resin. Fraction H1-F3, soluble in water and insoluble in 71 per cent (by volume) ethanol. Fraction H2-F1, soluble in water and insoluble in 63 per cent (by volume) ethanol. Fraction H2-F3, soluble in 63 per cent (by volume) ethanol and insoluble in 74 per cent (by volume) ethanol. Fraction H3-F1, soluble in water and insoluble in 63 per cent (by volume) ethanol. Fraction H3-F2, soluble in 63 per cent (by volume) ethanol and insoluble in 71 per cent (by volume) ethanol. Fraction H4-F1, soluble in water and insoluble in 71 per cent (by volume) ethanol.

\* Extinction curves were measured on solutions containing about 900  $\gamma$  of compound per ml. rather than the usual 300 to 350  $\gamma$ .

hog gastric mucin,<sup>3</sup> centrifuged free of inactive water-insoluble material, shows strong selective absorption in the ultraviolet at 260 m $\mu$  (Fraction C-135, Fig. 1) and appreciable apparent absorption even at 350 m $\mu$ . The latter absorption is due to light scattering, since mucin is visibly turbid and

<sup>3</sup> Wilson hog gastric mucin granules, item No. 443.

the apparent absorption extends far into the visible region where specific absorption is unlikely. The absorption above  $330\text{ m}\mu$  possesses a fairly constant Rayleigh coefficient of 2.5 to 3.5 over a considerable wave-length range, which presumably is typical for non-specific absorption (10, 11). The material absorbing at  $260\text{ m}\mu$  in mucin ("260  $\text{m}\mu$  component") is non-dialyzable, as may be seen from the data obtained with Fraction C-125.

The "260  $\text{m}\mu$  component" has not been adequately characterized, since it appears to be associated with the inactive or less active preparations isolated from mucin. The presence of nucleic acid is suggested by the wave-length of maximum absorption (12, 13), and Bray, Henry, and Stacey (14) have recently reported that mucin may contain nucleic acid of mixed ribose and desoxyribose type. However, preparations (0.04 per cent solutions) containing the "260  $\text{m}\mu$  component" did not give the Dische cysteine test (15) for desoxypentose. Weakly positive orcinol tests for pentose (16) were observed, but these results may not be significant, since hexoses, which are abundantly present in mucin, also are known to give weak orcinol tests (17). The "260  $\text{m}\mu$  component" cannot be removed by high speed centrifugation, since after centrifuging at 16,000 R.P.M. for 30 minutes (40,000*g*) the specific absorption remains. A slight decrease in extinction occurs throughout the ultraviolet owing to the separation of a small amount of suspended material and the resulting clarification of the solutions. Preliminary experiments indicate that the "260  $\text{m}\mu$  component" can be modified readily by heating with acid, alkali, or by the action of oxidizing agents such as hypiodite.

When an aqueous centrifuged mucin solution is fractionated with ethanol (2), the more active fractions which are soluble in 40 per cent ethanol and insoluble in 60 to 65 per cent ethanol always show considerably less absorption throughout the ultraviolet region than the original mucin solution (Fractions C-128 and C-143, Fig. 1; Fractions R4-F3 and R5-F3, Fig. 2). Although the "260  $\text{m}\mu$  component" is concentrated in the fractions insoluble in 40 per cent ethanol (Fractions C-123 and C-142, Fig. 1; Fractions R4-F2, and R5-F2, Fig. 2), those soluble in 40 per cent ethanol and insoluble in 60 to 65 per cent ethanol still contain significant amounts of the "260  $\text{m}\mu$  component" ( $E_{1\text{cm}}^{1\%} = 5$  to 6). The sensitivity and usefulness of the spectrophotometric method may be appreciated by the fact that while the above four more active fractions have essentially the same serological activity their extinction curves differ significantly. A new component is detectable in the fraction soluble in 60 to 65 per cent ethanol, since absorption occurs maximally in this case at about  $270\text{ m}\mu$  (Fraction R4-F4, Fig. 2). This fraction, which possesses essentially no serological activity, contains considerable quantities of free amino acids, and in one instance about 2 per

cent of amino acid nitrogen was detectable by the Van Slyke manometric method.

The products isolated from hog gastric mucin by Morgan and King (3) by 90 per cent phenol extraction followed by precipitation with ethanol or by aqueous extraction followed by precipitation with sodium sulfate were not distinguishable by a number of different tests including serological activity, elementary analysis, optical rotation, or viscosity. However, we

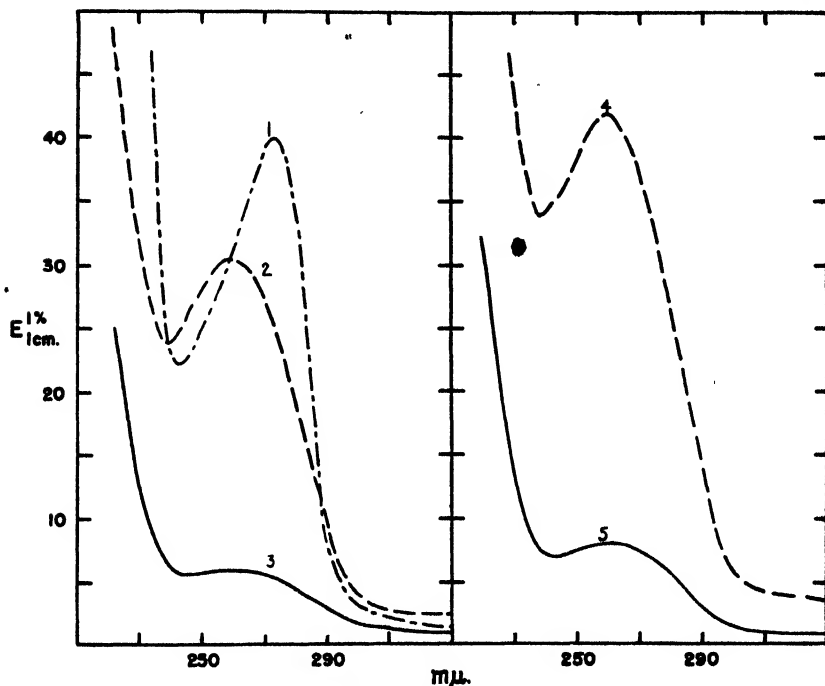


FIG. 2. Extinction curves of fractions isolated from hog gastric mucin by fractionation with ethanol. Curve 1, Fraction R4-F4; Curve 2, Fraction R4-F2; Curve 3, Fraction R4-F3; Curve 4, Fraction R5-F2; Curve 5, Fraction R5-F3.

have found that the extinction curves of fractions of group A substance prepared according to these procedures differ markedly and that the product from the phenol-ethanol procedure contains considerably greater amounts of the "260  $m\mu$  component" (Fraction C-52, Fig. 3) than the group A substance isolated either by ethanol or sodium sulfate fractionation (Fraction R2-F2A, Fig. 3).<sup>4</sup> It was reported earlier (5) that group A substance frac-

<sup>4</sup> The product isolated by the sodium sulfate procedure of Morgan and King (3) would be represented by an extinction curve roughly intermediate between Fractions R2-F2A and R2-F2C (Fig. 3), since the Morgan and King product was fractionated further as discussed above.

tions obtained by ethanol, sodium sulfate, or phenol-ethanol fractionation could be shown to be heterogeneous by electrodialysis. Solid material separated in the cell during electrodialysis, leaving a fairly clear supernatant. The residue and the material in the supernatant possessed approximately the same equivalent N-acetylglucosamine content and the same inhibition of isoagglutination activity. In the more precise inhibition of

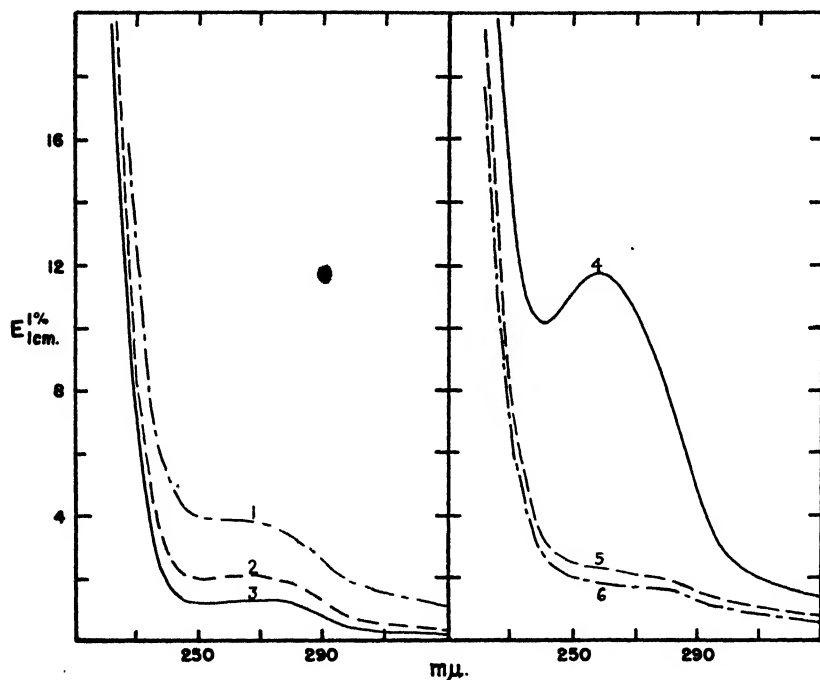


FIG. 3. Extinction curves of fractions isolated from hog gastric mucin by fractionation with sodium sulfate followed by electrodialysis (left-hand) and with phenol-ethanol (right-hand). Curve 1, Fraction R2-F2C; Curve 2, Fraction R2-F2B; Curve 3, Fraction R2-F2A; Curve 4, Fraction C-52; Curve 5, Fraction C-61; Curve 6, Fraction C-59.

hemolysis test the material in the supernatant appeared to be 20 to 25 per cent more active than the residue. While these two fractions are similar by the above criteria, they differ substantially in their spectral properties. The solid disperses in water to give quite turbid solutions, while the material in the supernatant yields only slightly turbid solutions. The latter solution, of course, scatters considerably less light in the ultraviolet, as may be seen from the extinction in the region 300 to 350  $m\mu$ . In Fig. 3, Fractions R2-F2A, R2-F2B, and R2-F2C are, respectively, the material in the supernatant, a washing of the residue, and the residue. Fractions R8-F1 and

R8-F2B (Fig. 4) are, respectively, the supernatant material and the residue obtained on electrodialysis of Fraction R5-F3 which was prepared by the ethanol fractionation of mucin. Fractions C-59 and C-61 (Fig. 3) are the soluble materials obtained on electrodialysis of the product of phenol-ethanol fractionation, Fraction C-52. Fractions C-59 and C-61 differ in that the former is soluble in 47 per cent ethanol and insoluble in 60 per cent

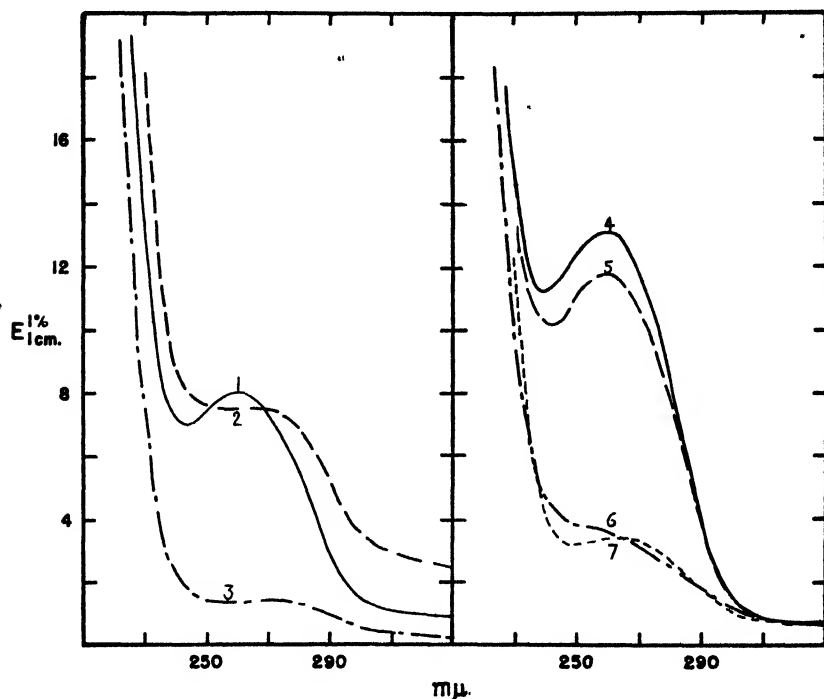


FIG. 4. Extinction curves of fractions isolated from hog gastric mucin by fractionation with ethanol followed by electrodialysis (left-hand) and by treatment with ion exchange resins (right-hand). Curve 1, Fraction R5-F3; Curve 2, Fraction R8-F2B; Curve 3, Fraction R8-F1; Curve 4, Fraction C-110; Curve 5, Fraction C-165; Curve 6, Fraction C-167; Curve 7, Fraction C-168.

ethanol, while the latter is insoluble in 47 per cent ethanol. In every case the soluble materials, which are more active serologically than the less soluble, or the parent, substances, show decreased absorption in the ultraviolet. This appears to be due to the fact that light-scattering components are concentrated in the residue and also because the "260  $m\mu$  component" occurs to a greater extent in the less soluble fractions.

A study has been made in this laboratory<sup>5</sup> of the use of ion exchange

<sup>5</sup> Unpublished experiments of E. L. Bennett.

resins for the preparation of group A substance from hog gastric mucin. Preparations comparable in activity to those isolated by other procedures were obtained in this way. Fraction C-168 (Fig. 4) which exhibits weak absorption in the ultraviolet was obtained by treating a 60 per cent ethanol precipitate from mucin twice with base-treated De-Acidite.<sup>6</sup> The 40 per cent ethanol precipitate, which contains considerable quantities of the "260 m $\mu$  component" (Fraction C-110), can be transformed to a fraction showing weak absorption at 260 m $\mu$  by treatment with base-treated De-Acidite (Fraction C-167, Fig. 4). Treatment of Fraction C-110 with base-treated Amberlite IR-4 resin,<sup>7</sup> a resin of similar type, is ineffective, however, in removing the "260 m $\mu$  component" (Fraction C-165, Fig. 4).

Group A substance is known to be degraded under a variety of conditions; e.g., by heating with formamide at 150° (2, 3), by digestion with papain-HCN (2), and by treatment with alkali (3, 9). Degradation is usually associated with a decrease in activity in the isoagglutination test and no change or an increase in activity in the hemolysis test. It was of interest to determine the extent to which the spectral properties of preparations of group A substance were altered by degradation. Typical curves are shown in Fig. 5. All of the degraded preparations show very little absorption in the ultraviolet and, as a consequence of the clarity of the aqueous solutions, low extinction values are observed in the 300 to 350 m $\mu$  range. Fraction R6-F5A was obtained from Fraction R4-F3 by papain-HCN digestion (2), Fraction R7-F5A by heating Fraction R5-F3 with formamide at 150° (2), and Fraction C-47 by preliminary digestion with 2 per cent sodium carbonate at 70°, followed by adsorption and precipitation according to the procedure of Meyer, Smyth, and Palmer (18).

*Extinction Curves of Preparations from Hog Pepsin*—Extinction curves for fractions isolated by the procedure of Landsteiner and Chase (19), Fraction C-22, and by the procedure of Landsteiner and Harte (2), Fraction C-26, from Wilson pepsin are given in Fig. 5. Unlike the active preparations from hog gastric mucin, the preparations obtained from pepsin show specific absorption in the region 270 m $\mu$ , which may be due to components derivable from the large quantities of protein present in the starting material. While these fractions are among the most active in the hemolysis test that have been isolated so far from hog stomach sources, evidence has been presented previously (5) which suggests that these preparations may be in fact partially degraded. The extinction curves would indicate that, despite their high potency in the hemolysis test, they contain contaminants or artifacts produced in the course of isolation which absorb at 270 m $\mu$ . Puri-

<sup>6</sup> The Permutit Company, New York.

<sup>7</sup> The Resinous Products and Chemical Company, Philadelphia, Pennsylvania.

fied preparations from hog gastric mucin show considerably less absorption in the ultraviolet.

*Extinction Curves of Preparations from Individual Hog Stomach Linings*—Witebsky (20) reported that only 40 per cent of the hogs examined have the specific blood group A factor in their stomach linings. It was later reported by Bendich, Kabat, and Bezer (21) that hog stomach linings may be differ-

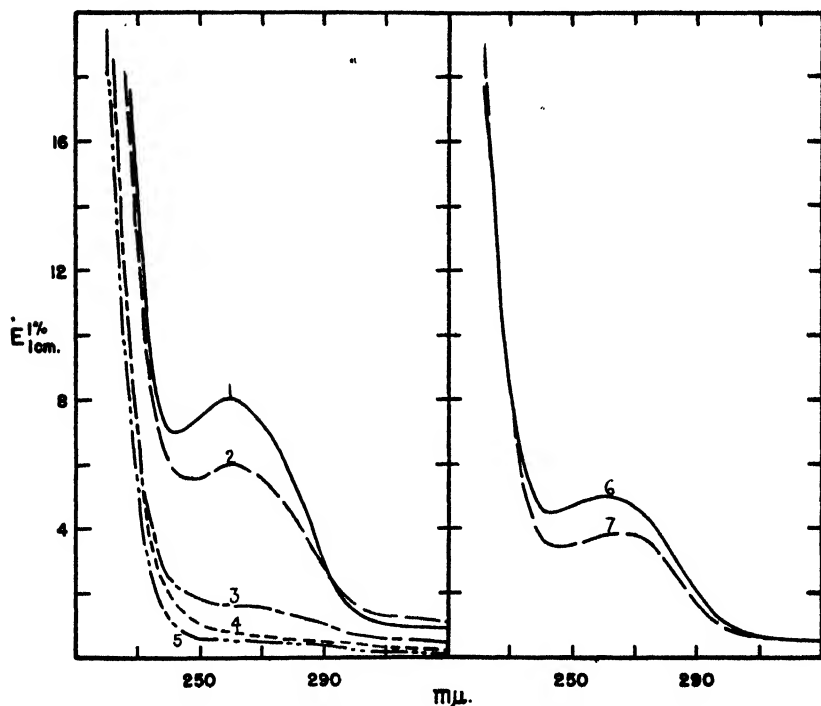


FIG. 5. Extinction curves of fractions isolated from hog gastric mucin by degradative procedures (left-hand) and from Wilson pepsin (right-hand). Curve 1, Fraction R5-F3; Curve 2, Fraction R4-F3; Curve 3, Fraction R7-F5A; Curve 4, Fraction C-47; Curve 5, Fraction R6-F5A; Curve 6, Fraction C-22; Curve 7, Fraction C-26.

entiated into those possessing the group A factor and those having a similar but ineffective mucoid. The latter material was shown by Aminoff, Morgan, and Watkins (22) to possess properties of blood group O substance. Hog gastric mucin, being a pool of stomachs of many hogs, was shown to contain both group A and O substances. It was of interest to compare the spectral properties of the group A substance derived from individual hog stomach linings with those isolated from hog mucin. Accordingly, partially purified fractions were isolated from individual hog stomach linings by



peptic digestion (21), followed by alcohol fractionation. The extinction curves for these preparations are shown in Fig. 6 and their serological properties in Table I. Hogs 1 and 4 gave inactive products, presumably group O substance, while Hogs 2 and 3 possessed group A-specific material. The activities of the more potent preparations were of about the same order of magnitude as those obtained from mucin and pepsin. However, all of the

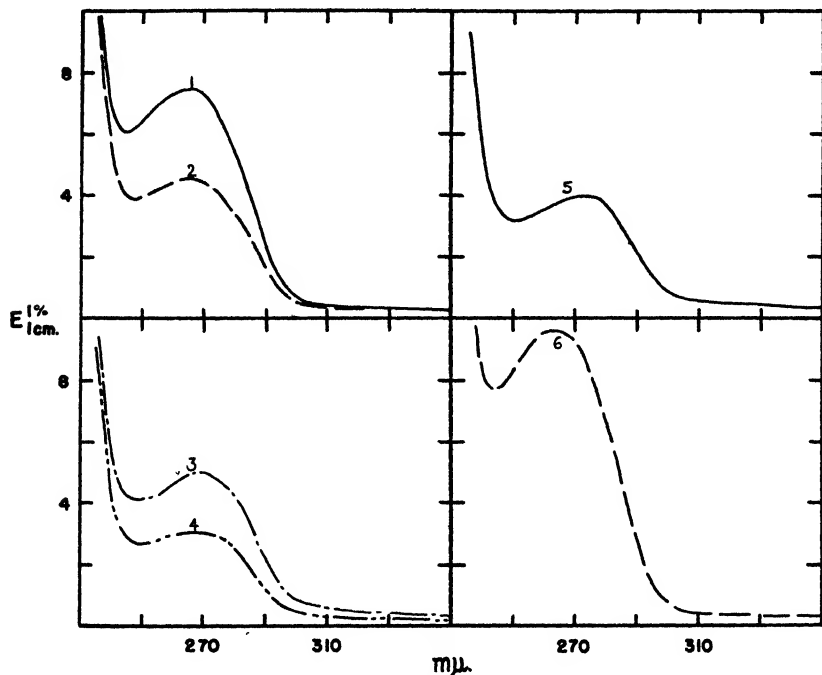


FIG. 6. Extinction curves of fractions isolated from individual hog stomach linings by fractionation with ethanol. Curve 1, Fraction H3-F1; Curve 2, Fraction H3-F2; Curve 3, Fraction H2-F1; Curve 4, Fraction H2-F3; Curve 5, Fraction H1-F3; Curve 6, Fraction H4-F1.

preparations showed considerably more specific absorption in the region 260 to 270  $m\mu$  than did comparable preparations derived from hog gastric mucin. While spectra of materials not possessing group A activity, Fractions H1-F3 and H4-F1, resemble those possessing group A activity, Fractions H2-F1 and H3-F1, it should be emphasized that specific absorption in the 260  $m\mu$  region is to be associated with the presence of inactive non-blood group-specific components.

Fractions H2-F3 and H3-F2 represent fractions not previously observed as arising from the fractionation of hog gastric mucosa. These fractions are soluble in 60 per cent ethanol and insoluble in 72 per cent ethanol and have little activity when compared to the fractions insoluble in 60 per cent

ethanol (Fractions H2-F1 and H3-F1). However, the relative potencies of Fractions H2-F3 and H3-F2 when compared with hog gastric mucin are about 10 to 20 times greater in the hemolysis test than in the isoagglutination test, a behavior similar to that observed for degraded preparations from mucin (2, 3, 5). Undegraded preparations from mucin have approximately equivalent relative activities in these tests. The degraded character of group A substance isolated from pepsin has been previously recognized (5), but the possibility of degradation of group A substance in the peptic digestion of hog stomach linings has not been appreciated.

*Maximum Tyrosine and Tryptophan Content of Preparations of Group A Substance*—The tyrosine or tryptophan content of group A substance preparations can be calculated by ascribing the net absorption at 280  $m\mu$  entirely to either tyrosine or tryptophan.<sup>8,9</sup> It is necessary to apply a correction for non-specific absorption (24, 25), and if this correction is applied throughout the ultraviolet region, then the more potent preparations which show low plateaus in the region 260 to 280  $m\mu$  possess extinction curves which have small maxima between 270 to 280  $m\mu$ . The maximum tryptophan and tyrosine contents of both Fractions R6-F5A and C-47, which are among the most transparent in the ultraviolet, were calculated in this way to be 0.1 and 0.4 per cent, respectively. Undegraded preparations with less transparency would have correspondingly higher values, *i.e.*, 0.2 to 0.3 per cent tryptophan and 0.8 to 1.2 per cent tyrosine. These maximum estimates would of course be lower if both amino acids were present. While these preparations are probably not pure, it seems certain that tyrosine and tryptophan, if indeed present in group A substance, are among the least abundant amino acids. The above results are consistent with the observation that qualitative tests for the aromatic amino acids in group A substance are generally weak or negative (2) and with the sole quantitative result of Brand and Saidel (27) to the effect that a preparation of group A substance obtained from hog gastric mucin by the phenol procedure contained 0.3 per cent tyrosine and 0.2 per cent tryptophan.

#### SUMMARY

A study of the ultraviolet absorption spectra of preparations of blood group A-specific substance isolated from hog gastric mucin supports the

<sup>8</sup>  $E_{1\%}^{1\text{cm}}$  for tyrosine and tryptophan at 280  $m\mu$  are 69 and 278, respectively, in phosphate-citrate buffer at pH 7.2. Phenylalanine shows weak absorption at 255  $m\mu$ , and histidine shows specific absorption only below 240  $m\mu$ . Sugars would be expected to absorb only below 230  $m\mu$  (13, 23).

<sup>9</sup> Since group A substance preparations absorb weakly in the ultraviolet and because tyrosine and tryptophan possess similar absorption spectra under the test conditions, it is difficult to determine tyrosine and tryptophan simultaneously as has been done previously (24-26). The procedure of measuring absorption in alkaline solution which allows considerable differentiation in the tyrosine and tryptophan spectra is inapplicable due to the lability of group A substance under these conditions.

view that group A substance, and probably group O substance, has little specific absorption in this spectral region and that other components account for most if not all of the specific absorption noted in the case of impure preparations. However, ultraviolet spectrophotometric analyses have been found to be particularly useful in following changes in composition brought about by the fractionation of group A substance preparations obtained from hog gastric mucin as well as in comparing preparations isolated by different methods and from different sources. Such analyses have also indicated that tyrosine and tryptophan, if indeed present in group A substance, are but minor constituents.

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## FOLIC ACID ACTIVITY IN HOMOGENATES OF RAT LIVER\*

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It has been demonstrated that homogenates of rat liver increase in folic acid activity following incubation at pH 7 (1, 2). In 1944, Mims, Totter, and Day (3) used a partially purified enzyme preparation from rat liver in determining "substances convertible into the factor stimulating *Streptococcus lactis* R," and in the same year Binkley *et al.* (4) discovered vitamin B<sub>9</sub> conjugate, stating that its presence in certain liver extracts had been demonstrated. Laskowski, Mims, and Day (5) later found that rat liver extracts showed vitamin B<sub>9</sub> conjugase activity. It appeared probable, therefore, that the increased folic acid content of rat liver homogenates following incubation at pH 7 was the result of the enzymatic conversion of the conjugate to folic acid. However, the increase in folic acid activity of the liver homogenates incubated at pH 7 was greater than that in homogenates incubated at pH 5 (2), suggesting that the conjugase in rat livers differs from similar enzymes in the livers of hogs and chickens, the activity of the latter being enhanced at the lower pH levels (6). This explanation is not borne out by the studies discussed here, for the activity of rat liver conjugase has been found to be greater at pH 5 than at pH 7. Thus it appears that the increases in the folic acid content of the liver homogenates incubated at pH 7 were not the result of the enzymatic conversion of the conjugate to PGA (pteroylglutamic acid). Data are presented which indicate that rat liver contains a substance, other than PGA conjugate, subject to enzymatic conversion to folic acid or substances showing folic acid activity.

### Methods

The folic acid activity was determined by using either *Streptococcus faecalis* and the turbidimetric method of Luckey, Briggs, and Elvehjem (7) (the medium was modified by the addition of Salts B (8)) or *Lactobacillus casei* and the acidimetric method of Teply and Elvehjem (8) (the medium

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was modified by omission of norit-treated peptone). Synthetic pteroylglutamic acid, supplied by the Lederle Laboratories Division, American Cyanamid Company, was used as a standard in all cases.

Rat livers removed from normal, mature rats and frozen in the freezing compartment of a refrigerator were used in all experiments. Homogenates were prepared in a Waring blender by grinding 3 gm. of the frozen liver in 50 ml. of distilled water for  $1\frac{1}{2}$  minutes. After grinding, the homogenate was allowed to stand for about 1 minute so that the foam could rise, and then samples were removed by pipetting.

#### EXPERIMENTAL

The rate of increase in folic acid activity of rat liver homogenate incubated at two different pH levels was studied first. Two mixtures, one

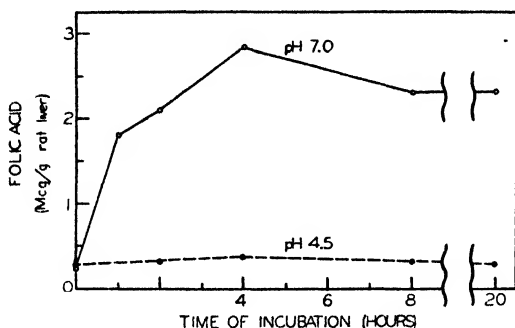


FIG. 1. Folic acid content of rat liver homogenates incubated at pH 4.5 and 7.0

containing 20 ml. of freshly prepared rat liver homogenate and 10 ml. of 0.1 M phosphate buffer, pH 7.0, the other 20 ml. of the homogenate and 10 ml. of 1 per cent sodium acetate buffer, pH 4.5, were used. Toluene was added to each mixture to inhibit bacterial growth. Immediately after the addition of the homogenate to the buffer and toluene, 3 ml. samples of the mixtures were pipetted into 18 mm. test-tubes, which were placed in a boiling water bath. After 2 minutes these initial samples were removed from the bath, allowed to cool, and placed in the freezing compartment of a refrigerator. The remaining reaction mixtures, in stoppered 125 ml. Erlenmeyer flasks, were placed in an air incubator at  $37^{\circ}$ . At 1, 2, 4, 8, and 20 hours, 3 ml. samples were removed and treated in the same manner as were the zero time samples. After all the samples had been collected, they were diluted, neutralized to pH 7.0, made up to volume, and filtered. The filtrates were assayed with *Streptococcus faecalis* and the results obtained are shown in Fig. 1. The data obtained with the 20 hour samples agree with results obtained by Burkholder, McVeigh, and Wilson (2) on rat liver homo-

genates incubated at pH 7.0 and 5.0 for a similar period of time. In the liver homogenates incubated at pH 7.0, the rapid increase in folic acid and the subsequent decrease agree in general with results obtained by Wright, Skeggs, and Welch (1).

In an experiment similar to the one just discussed, it was demonstrated that the failure of the liver homogenate to increase in folic acid at pH 4.5 was not the result of acetate inhibition. Five incubation mixtures were used. Each contained 20 ml. of fresh homogenate and 10 ml. of buffer or water, as indicated in Fig. 2. In this experiment, when samples were withdrawn, they were added to 12 ml. of a buffer medium of such composition that the resulting mixtures were identical in buffer content. After thus equalizing the buffers, the samples were heated in a boiling water bath for 3

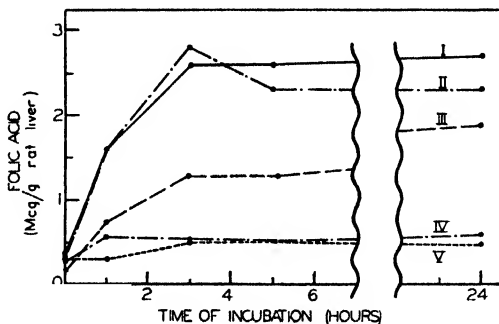


FIG. 2. Folic acid in liver homogenates incubated in various buffers. Curve I, ethylenediamine-citrate buffer, final pH 7.0; Curve II, citrate-phosphate buffer, final pH 7.0; Curve III, water, final pH 6.3; Curve IV, citrate-phosphate buffer, final pH 4.6; and Curve V, sodium acetate buffer, final pH 4.3.

minutes, cooled, refrigerated, and assayed, as already described. The results obtained (Fig. 2) are in agreement with those obtained in the first study, and they indicate that the differences obtained were not the result of the use of different kinds of buffers or of heating the samples at the different hydrogen ion concentrations.

We next studied the effect of pH on the release of folic acid from the homogenate and on the conjugase activity of the liver. To determine the optimum pH for the release of the vitamin from the liver, 5 ml. samples of homogenate were added to 18 mm. test-tubes containing 10 ml. of 0.1 M citrate-phosphate buffer of various hydrogen ion concentrations. In this experiment, the homogenate and buffer solutions were at 37° when mixed. After incubating for 1 hour at 37°, the test-tubes were placed in a boiling water bath for 3 minutes and then cooled to room temperature. 5 ml. samples were withdrawn, diluted to about 25 ml., neutralized to pH 7.0, and made up to 100 ml. volume. After filtering, the solutions were assayed

with *Streptococcus faecalis*. The sample remaining after withdrawal of 5 ml. for assay was used for a pH determination.

The optimum pH for rat liver conjugase was determined as follows: 5 ml. of 0.1 M citrate-phosphate buffer of various hydrogen ion concentrations

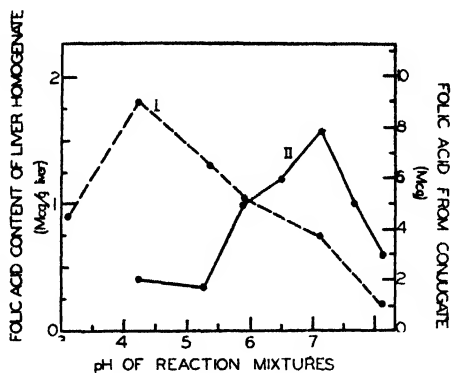


FIG. 3. Effect of pH on the folic acid formed in rat liver homogenate upon incubation and on the conjugase activity of the homogenate. Curve I, folic acid produced from PGA conjugate; Curve II, folic acid produced from the homogenate.

TABLE I

*Inability of Hog Kidney Conjugase to Increase Folic Acid Content of Heated Rat Liver Homogenate*

Reaction mixture	Folic acid content, <i>Streptococcus faecalis</i>
	$\gamma$
5 ml. rat liver homogenate, 5 ml. citrate-phosphate buffer, pH 7.0, heated*	0.065
5 ml. rat liver homogenate, 5 ml. sodium acetate buffer, pH 4.5	0.065
5 ml. rat liver homogenate, 5 ml. sodium acetate buffer, pH 4.5, heated,* 1 ml. hog kidney conjugase	0.065†
5 ml. rat liver homogenate, 5 ml. citrate-phosphate buffer, pH 7.0	0.58
1 ml. hog kidney conjugase, 5 ml. sodium acetate buffer, pH 4.5	0.015

\* Heated 3 minutes in a boiling water bath.

† Hog kidney conjugase blank subtracted.

and 3 ml. of solution containing 10  $\gamma$  of pteroylglutamic acid as PGA conjugate (crystalline conjugate supplied by Parke, Davis and Company) were added to a series of 18 mm. test-tubes. A rat liver homogenate was diluted with 4 volumes of water and 2 ml. were then added to each test-tube. All solutions were at 37° when mixed. After incubating for 1 hour at 37°, the test-tubes were heated for 3 minutes in a boiling water bath and cooled. A 1 ml. sample was withdrawn for assay and the remainder of the sample

was used for the pH determination. The results of this and the previous study are given in Fig. 3. Although rat liver conjugase did function at the same pH at which the increases in folic acid in the incubated homogenates were the largest, the conjugase activity was greatest in the more acid mixtures in which no increases in the folic acid content of the incubated mixtures were found (see Fig. 1 also). It appears, therefore, that the precursor of the folic acid formed in the homogenates is not PGA conjugate but some other substance of low microbiological activity which can be enzymatically converted to the vitamin.

Further, it was found that hog kidney conjugase would release no folic acid from heated liver homogenate. 5 ml. of the freshly prepared homogenate were measured into each of four test-tubes and treated as indicated

TABLE II  
*Ability of Rat Liver Homogenate to Increase Folic Acid Content of Heated Liver Homogenate*

Reaction mixture*	Folic acid content, <i>Streptococcus faecalis</i> <sup>†</sup>
	$\gamma$
5 ml. heated† rat liver homogenate,‡ 5 ml. citrate-phosphate buffer, pH 7.0	0.09
5 ml. rat liver homogenate,‡ 5 ml. citrate-phosphate buffer, pH 7.0	0.88
5 ml. heated† rat liver homogenate,‡ 5 ml. rat liver homogenate‡	1.73

\* Incubated under toluene at 37° for 4 hours.

† Heated 3 minutes in a boiling water bath.

‡ Homogenate prepared as usual, except that pH 7.0 citrate-phosphate buffer was used in place of water.

in Table I. The reaction mixtures listed were incubated under toluene at 37° for 20 hours and assayed with *Streptococcus faecalis*, as described before. The conjugase used was a frozen water extract of hog kidney prepared as described by Bird *et al.* (9). Its activity was demonstrated on both crystalline PGA conjugate and yeast extract before its use in this experiment. The failure of the hog kidney enzyme to increase the folic acid content of the heated homogenate is further evidence that the precursor is not the regular PGA conjugate. The data in Table II demonstrate that the precursor in the heated liver homogenates can be converted to folic acid by the liver enzyme.

Since it appeared possible that the vitamin might be bound to proteins, making it unavailable to the test organisms, a sample of cold liver homogenate was adjusted to pH 5.0 with acetic acid and centrifuged. The supernatant liquid was adjusted to pH 7.0 and again centrifuged. This supernatant



liquid was treated with solid ammonium sulfate (8 gm. per 15 ml.) and the precipitate which formed was centrifuged off and resuspended in pH 7.0 citrate-phosphate buffer. Although the fraction precipitated by ammonium sulfate contained about 25 per cent of the folic acid precursor present in the original liver sample (as determined by assay following incubation with rat liver homogenate), all of the precursor was removed by dialysis overnight against cold running water. Dialysis of the solution prior to ammonium sulfate treatment gave similar results. It was therefore concluded that, if the substance is bound to proteins, the binding is a very loose one. Very little of the precursor could be found in the precipitates formed at pH 4.5 and 7.0.

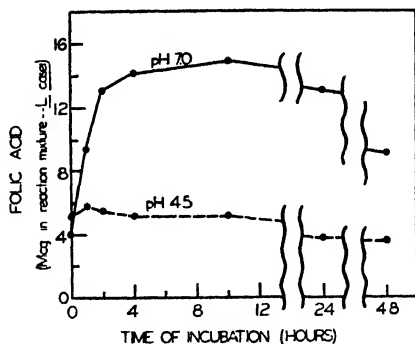


FIG. 4. Folic acid in mixtures of liver extract and homogenate incubated at two hydrogen ion concentrations.

We next ground 100 gm. of frozen liver with 200 ml. of cold water for 2 minutes in a Waring blender. 25 ml. of cold 5 per cent sodium acetate buffer, pH 4.5, were then added and the mixture was ground again for 2 minutes. The addition of the buffer brought the pH of the mixture to 5.3; our previous findings indicated that enzymatic conversion of the precursor to folic acid at this pH was very slow. The mixture was then transferred to a 600 ml. beaker in a boiling water bath and stirred continually until it reached a temperature of 90°. It was maintained at this temperature for 5 minutes and then cooled. After filtering with gentle suction through a filter paper, the residue was mixed with about 50 ml. of water and again sucked dry on the filter. The combined filtrate and washings were concentrated *in vacuo* to about 75 ml., neutralized to pH 7.0, and made up to 100 ml. Samples taken before and after incubation with fresh liver homogenate were assayed for folic acid with *Streptococcus faecalis*. The following data were obtained: folic acid plus the precursor in original liver, 220  $\gamma$ ; folic acid in filtrate, 39  $\gamma$ ; and folic acid as the precursor in filtrate, 93  $\gamma$ . Whereas only 60 per cent of the folic acid plus the precursor present in the original

liver sample was recovered in the filtrate, the method yielded a solution of substrate (folic acid precursor) free of most of the protein present in the liver homogenate. This substrate was incubated at 37° under toluene in the presence of freshly prepared liver homogenate at pH 4.5 and 7.0; the mixtures were sampled for assay at 0, 1, 2, 4, 10, 24, and 48 hours. In this experiment 15 ml. each of liver extract, homogenate, and buffer (0.1 M citrate-phosphate, pH 7.0, or 1 per cent sodium acetate, pH 4.5) were used in each mixture. Samples were withdrawn and prepared for assay as described earlier, except that filtering was omitted. *Lactobacillus casei* was used in the assay (titrimetric) instead of *Streptococcus faecalis*. The results of the study (Fig. 4) are very similar to those obtained in the incubation of rat liver homogenate alone. Here, however, the disappearance of folic acid following the initial increase is more clearly apparent as the time of incubation is lengthened. These data indicate that adsorption and occlusion are not responsible for the differences in the release of folic acid from homogenates incubated at pH 4.5 and 7.0.

#### DISCUSSION

In the microbiological assay of various liver extracts with *Streptococcus faecalis*, the response of the microorganism to synthetic PGA was not the same as it was to the liver extracts. Decreasing responses with the addition of increasing amounts of the liver extracts (as compared to the responses to PGA) caused "drifts." This introduced errors into the assay which were in part overcome by selecting values from a limited part of the standard curve only. By doing this, the results obtained in the assay of several solutions of liver extracts with *S. faecalis* compared well with the results obtained with *Lactobacillus casei* (titrimetric). "Drifts" were not apparent with the latter microorganism with which longer incubation periods were used. The occurrence of "drift" indicated that *S. faecalis* was responding to substances other than PGA, and we therefore chose to use the term "folic acid" in expressing our results. Piffner *et al.* (10) reported the presence of an acid-labile factor in horse liver to which *S. faecalis* responded differently than did *Lactobacillus casei*. It is possible that the occurrence of such a substance in rat liver after incubation would cause "drifts" such as we have observed.

When Binkley *et al.* (4) reported the presence of vitamin B<sub>9</sub> conjugate in certain liver extracts, they did not state the method by which it was determined. Our work indicates that the conjugate is present in rat livers in small amounts only. However, the possibility that the precursor which we have observed is a larger molecule, which is first degraded to a conjugate and subsequently converted to PGA, still exists, since even at pH 7.0 the liver conjugase is quite active.

Our findings suggest that certain liver preparations may be of even greater value than hog kidney or chicken pancreas conjugases for enzymatically treating samples prior to microbiological assay. However, crude liver preparations apparently cause some destruction of folic acid and this property may limit their use. Partially purified liver preparations may prove valuable.

#### SUMMARY

Rat liver homogenates incubated at pH 7.0 increased rapidly in their folic acid content, whereas those incubated at pH 4.5 did not. The optimum pH was between 4.0 and 5.0 for folic acid conjugase in the homogenates. Since at pH 4.5 neither autolysis of the liver nor digestion of heated samples with hog kidney conjugase caused any liberation of the vitamin, the presence of a precursor of folic acid other than pteroylglutamic acid conjugate is indicated. This precursor is dialyzable and stable to short heat treatments.

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# SOME STRUCTURAL ANALOGUES ANTAGONISTIC TO PTEROYLGLUTAMIC ACID

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In order to extend the studies made in this laboratory (1) on structural analogues antagonistic to metabolically important compounds, a number of possible inhibitory analogues of pteroylglutamic acid have been prepared and tested biologically. Since this vitamin is a pyrimidine compound, the prediction could be made on the basis of existing knowledge (2, 3) that replacement of this part of the molecule with a benzene ring would yield an antagonist. Attempts were therefore made to synthesize quinoxaline-2-(*p*-methyleaminobenzoylglutamic acid) by the condensation of *o*-phenylenediamine, dibromopropionaldehyde, and *p*-aminobenzoylglutamic acid according to the general procedure (4) used in the formation of pteroylglutamic acid. An active growth inhibitor was produced in the reaction and obtained in relatively pure form, but it did not appear to be the expected quinoxaline derivative, but rather a more complex substance.

A slightly different type of inhibitory analogue was secured by the formation of quinoxaline-2-carboxyl-*p*-aminobenzoylglutamic acid. This compound was readily obtainable in pure form and was found to be a rather special kind of antifolic acid in that it was effective in only one species.

The most powerful antagonist examined in this investigation was 2-amino-4,7-dihydroxypteridine-6-carboxyl-*p*-aminobenzoylglutamic acid. This substance is the same as pteroylglutamic acid, except that the methylene bridge has been exchanged for a carbonyl group, and the hydrogen at position 7 has been replaced by OH. It was thus a derivative of isoxanthopterincarboxylic acid. The corresponding xanthopterincarboxylic acid derivative was prepared and found to be much less potent.

Analogues identical with pteroylglutamic acid, except for the replacement of glutamic by aspartic acid or by methionine sulfoxide, were prepared and shown to be antagonistic to the vitamin in bacterial growth. However, others (5, 6) have recently published reports dealing with the aspartic acid compound, and therefore it will not be described here.

## EXPERIMENTAL

*Compound Derived from o-Phenylenediamine, Dibromopropionaldehyde, and p-Aminobenzoylglutamic Acid*—A solution of 13.3 gm. of *p*-amino-

benzoylglutamic acid and 5.4 gm. of *o*-phenylenediamine in 500 cc. of 0.1 M acetate buffer at pH 4 was stirred and treated dropwise with a solution of dibromopropionaldehyde prepared by adding 2.7 cc. of bromine to 3.3 cc. of acrolein in 50 cc. of ether in the cold. The product began to precipitate immediately and was filtered after standing overnight in the refrigerator. The crude product (10 gm.) was suspended in 500 cc. of 2 N sodium hydroxide solution. The insoluble material was discarded, the filtrate adjusted to pH 7 with dilute hydrochloric acid, and chromatographed on 250 gm. of aluminum oxide. The column was washed with 200 cc. of water and, after concentrating the effluent to 500 cc., it was adjusted to pH 1 with concentrated hydrochloric acid. The precipitate was discarded. The active material (0.6 gm.) precipitated from the filtrate when the pH was raised to 4 with 5 N sodium hydroxide solution. Further purification was accomplished by a counter-current distribution (7) of 1 gm. between 100 cc. portions of butanol and a 3 M aqueous pyridine solution. The active material was most abundant in the third of a series of nine tubes. Repetition of the counter-current distribution indicated the substance to be homogeneous. During the entire purification the antagonist was followed by microbiological tests with *Lactobacillus casei*. The major part of the potency of the crude reaction mixture was obtained in the final product.

Analyses showed C 61.2, H 5.6, N 12.1, and glutamic acid (8) 21. Although this compound appeared to be pure, it was not the desired quinoxaline, since theoretical values for  $C_{21}H_{20}O_5N_4$  are C 61.8, H 4.9, N 13.7, and glutamic acid 36.

*Quinoxaline-2-carboxyl-yl-p-aminobenzoylglutamic Acid*—To a cooled solution of 4.2 gm. of *p*-aminobenzoylglutamic acid and 4 gm. of sodium hydroxide in 200 cc. of water were added with stirring and cooling 8 gm. of quinoxaline-2-carboxyl-yl chloride (9) in 100 cc. of ether and 2 gm. of sodium hydroxide in 20 cc. of water. The acid chloride and the sodium hydroxide were added alternately in small portions. The ether layer was separated and the water layer was acidified to pH 4 with concentrated hydrochloric acid. The product (10 gm.) melted at 128°. Two recrystallizations from alcohol raised the melting point to a constant value of 145°. The compound was a pale yellow crystalline substance sparingly soluble in  $H_2O$ .

$C_{21}H_{18}O_5N_4 \cdot H_2O$ .	Calculated.	C 57.3,	H 4.5,	N 12.7,	glutamic acid (8) 33
	Found.	" 58.0,	" 4.6,	" 12.9,	" " 31

The water of hydration was not lost upon heating at 100° for 1 hour.

*2-Amino-4,7-dihydroxypteridine-6-carboxyl-yl-p-aminobenzoylglutamic Acid*—1.1 gm. of isoxanthopterincarboxylic acid prepared according to the

method of Purrmann (10) were triturated in a mortar with 2 gm. of phosphorus pentachloride and 30 cc. of phosphorus oxychloride. The mixture was allowed to stand for 2 days at room temperature and was then freed of phosphorus oxychloride by evaporation under reduced pressure and extraction with dry petroleum ether. The crude acid chloride was condensed at 0° with a cold solution of 2 gm. of *p*-aminobenzoylglutamic acid dissolved in 20 cc. of 1 N sodium hydroxide. The reaction was maintained alkaline by frequent additions of sodium hydroxide. After 2 hours hydrochloric acid was added to adjust the pH to 8, and a precipitate was separated by centrifugation and discarded. The solution was adjusted to pH 3 to 4 with hydrochloric acid or with acetic acid, and the orange precipitate which formed was collected and washed with water and then with alcohol. In some preparations this product contained isoxanthopterincarboxylic acid and this could be removed by taking advantage of the insolubility in water of its sodium salt. Occasionally, difficulty was experienced in the formation of the acid chloride because of the insolubility of isoxanthopterincarboxylic acid. The acid chloride could be formed by heating the dry acid with dry phosphorus pentachloride in an oil bath at 100–120°; but even with this method, complete conversion was not obtained. Apparently replacement of the hydroxyl groups at positions 4 and 7 did not occur, since the final product contained only a trace of halogen. In precipitation of the condensation product with acid, care was exercised to avoid a low pH because glutamic acid was readily lost under such conditions (see the next section). The material could be obtained crystalline by very cautious acidification of a solution of the sodium salt, but this was not a practical procedure for the preparation of large amounts of crystals. The substance did not melt at 300°.

$C_{15}H_{17}O_5N_7$ .	Calculated.	N 20.8,	glutamic acid 31
	Found.	" 21.0,	" " 30

*2-Amino-4,7-dihydroxypteridine-6-carboxyl-*p*-aminobenzoic Acid*—When an aqueous solution of the sodium salt of the analogue described in the previous paragraph was poured into boiling 1 N hydrochloric acid an orange precipitate formed which was apparently 2-amino-4,7-dihydroxypteridine-6-carboxyl-*p*-aminobenzoic acid. This followed from the analyses which showed no glutamic acid and 25.0 per cent nitrogen (theory, 24.6). The substance was obtained as well formed crystals by adding alcohol to a saturated acetic acid solution, but this was not a practical method because of the low solubility of the compound in acetic acid. It did not melt at 300°. The instability of the glutamic acid residue in the analogue described in the previous section was illustrated further by the finding that boiling an aqueous suspension of the compound for 10 minutes reduced the glutamic acid content to 10 per cent.

**Method of Biological Test with Bacteria**—In order to measure any antagonistic relationship of the compounds to pteroylglutamic acid, the medium described by Landy and Dicken (11) free of folic acid was used. For most of the tests this medium was fortified with 0.0001  $\gamma$  of pteroylglutamic acid<sup>1</sup> per cc. and *Lactobacillus casei* was employed. Other species were examined under similar conditions to determine the range of effectiveness of the compounds as growth inhibitors, and graded levels of the vitamin were studied in experiments to establish whether the antagonism with pteroylglutamic acid was competitive in nature.

Graded amounts of a substance to be tested were added to a series of tubes containing a constant amount of the vitamin plus the basal medium. After inoculation, and incubation for 48 hours, the amount of growth in

TABLE I

*Inhibitory Effect of 2-Amino-4,7-dihydroxypteridine-6-carboxylyl-p-aminobenzoylglutamic Acid on Lactobacillus casei in Presence of 0.0001  $\gamma$  of Pteroylglutamic Acid per Cc.*

Compound  $\gamma$ per cc.	Turbidity*
0	71
0.5	80
1.0	88
2.0	93
5.0	96

\* Expressed as the per cent of incident light transmitted by the culture in comparison to the uninoculated medium.

each tube was determined both turbidimetrically and by measurement of pH, according to the usual procedures. Curves were then constructed which related the concentration of the inhibitor to the amount of growth, and from these the concentration required to cause half maximal inhibition of growth was estimated. A typical response of *Lactobacillus casei* to graded amounts of 2-amino-4,7-dihydroxypteridine-6-carboxylyl-p-aminobenzoylglutamic acid is illustrated by the data in Table I.

**Inhibition of Growth of *Lactobacillus casei* by Structural Analogues of Pteroylglutamic Acid**—Table II contains data to show that the growth of *Lactobacillus casei* in the presence of 0.0001  $\gamma$  of pteroylglutamic acid per cc. was inhibited by most of the compounds related in structure to the vitamin. The analogues varied in potency, the most active being 2-amino-4,7-dihydroxypteridine-6-carboxylyl-p-aminobenzoylglutamic acid.

**Competition between Inhibitors and Vitamin**—The growth-inhibitory action of each of the compounds was completely erased by increases in the

<sup>1</sup> We wish to thank Dr. E. L. R. Stokstad and Dr. T. H. Jukes of the Lederle Laboratories Division, American Cyanamid Company, for gifts of this material.

TABLE II

Amounts of Analogues Required for Half Maximal Inhibition of Growth of Various Microbial Species in Presence of 0.0001  $\gamma$  of Pteroylglutamic Acid per Cc.

Compounds	Amounts required for half maximal inhibition for							
	<i>Lactobacillus casei</i>	<i>Streptococcus faecalis</i> R	<i>Lactobacillus arabinosus</i>	<i>Leuconostoc mesenteroides</i>	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Endomyces versatilis</i>	<i>Saccharomyces cerevisiae</i>
	$\gamma$ per cc.	$\gamma$ per cc.	$\gamma$ per cc.	$\gamma$ per cc.	$\gamma$ per cc.	$\gamma$ per cc.	$\gamma$ per cc.	$\gamma$ per cc.
2-Amino-4,7-dihydroxypteridine-6-carboxyl- <i>p</i> -aminobenzoyleglutamic acid	0.7	11	No effect at 50	No effect at 50	No effect at 50			
2-Amino-4,6-dihydroxypteridine-7-carboxyl- <i>p</i> -aminobenzoyleglutamic acid	2.5							
2-Amino-4,7-dihydroxypteridine-6-carboxyl- <i>p</i> -aminobenzoic acid	0.8							
Isoxanthopterin-carboxylic acid	No effect at 500							
Unknown quinoxaline from <i>o</i> -phenylenediamine	0.8	No effect at 500	No effect at 500	No effect at 500	No effect at 500	No effect at 500	No effect at 500	No effect at 500
Quinoxaline-2-carboxyl- <i>p</i> -aminobenzoyleglutamic acid	30	"	"	"	"	"	"	"
Quinoxaline-2-carboxylic acid	No effect at 1000							
Requirement for pteroylglutamic acid	Required	Required	None	None	None	None	None	None



concentration of pteroylglutamic acid. Furthermore, the antagonism was competitive in nature. The data in Table III will demonstrate this fact in the case of 2-amino-4,7-dihydroxypteridine-6-carboxyl-*p*-aminobenzoylglutamic acid. Here it can be seen that, except for the smallest concentration of the vitamin, when the amount of the growth factor was doubled, the amount of inhibitor required to reduce growth to half maximum likewise was doubled.

*Relative Effectiveness of Various Analogues on Number of Species*—The data in Table II show the concentrations of each of the analogues which were required to cause the half maximal inhibition of growth of each of a number of bacterial species when tested in the presence of 0.0001  $\gamma$  of pteroylglutamic acid per cc. In contrast to the situation recently described for other types of antifolic acids (5, 6, 12), most of the present substances were more effective against *Lactobacillus casei* than against *Streptococcus*

TABLE III

*Amounts of 2-Amino-4,7-dihydroxypteridine-6-carboxyl-*p*-aminobenzoylglutamic Acid Required for Half Maximal Inhibition of Growth of Lactobacillus casei in Presence of Varying Amounts of Pteroylglutamic Acid*

Pteroylglutamic acid	Analogue required for half maximal inhibition
$\gamma$ per cc.	$\gamma$ per cc.
0.0001	0.7
0.0002	2.5
0.0004	5
0.0008	9
0.0016	24

*faecalis*. In fact, some analogues seemed to be harmful only to *Lactobacillus casei*. Throughout the series the dependence of an inhibitory action on the organism's nutritional requirement for pteroylglutamic acid was apparent (cf. (13)).

*Antagonism between 2-Amino-4,7-dihydroxypteridine-6-carboxyl-*p*-aminobenzoylglutamic Acid and Pteroylglutamic Acid in Rats*—Copenhagen strain rats weighing approximately 125 gm. were fed on bread and milk, and injected intraperitoneally with solutions to be tested. A single dose of 10 mg. of 2-amino-4,7-dihydroxypteridine-6-carboxyl-*p*-aminobenzoylglutamic acid proved fatal within 24 hours, whereas 1 mg. per day for a week was tolerated. Simultaneous administration of 10  $\gamma$  of pteroylglutamic acid protected the animals against 10 mg. of the analogue. On the other hand, rats of this breed and weight tolerated daily intraperitoneal doses of 100 mg. of quinoxaline-2-carboxyl-*p*-aminobenzoylglutamic acid (as the sodium salt) for at least a week.

## SUMMARY

Several structural analogues of pteroylglutamic acid have been prepared for the first time and shown to inhibit the growth of certain bacteria competitively with the vitamin. 2-Amino-4,7-dihydroxypteridine-6-carboxyl-*p*-aminobenzoylglutamic acid was the most active of these, but quin-oxaline-2-carboxyl-*p*-aminobenzoylglutamic acid and a substance formed by the reaction of dibromopropionaldehyde with *o*-phenylenediamine and *p*-aminobenzoylglutamic acid likewise were effective against *Lactobacillus casei*. Of the microorganisms tested, only those which required pteroylglutamic acid as a growth factor were prevented from multiplying by the presence of the analogues.

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## ISOLATION AND ANTIFUNGAL ACTION OF NATURALLY OCCURRING 2-METHOXY-1,4-NAPHTHOQUINONE\*

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Several antibiotic compounds have been isolated from green plants. Those which have been structurally identified are citrinin (1-4), dicumarol (5-7), and protoanemonin (8, 9). Other less well defined factors are an oil from *Allium sativum* (10-12), a colorless crystalline compound from *Arctium minus* (13), Products A and B from *Asarum canadense* (14), cassic acid from the leaves of *Cassia reticulata* (15), crepin from the buds and flowers of *Crepis taraxacifolia* (16), a low melting compound from the leaves and flowers of *Spiraea aruncus* (17), and tomatin, occurring in *Lycopersicon esculentum* (garden tomato) (18). An antibacterial substance has also been extracted from *Tillandsia usneoides* (Spanish moss) (19), and the milky juice of crushed water chestnuts has been shown to inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, and *Aerobacter aerogenes* (20).

Many of these materials have been tested for activity exclusively against strains of the common test bacteria used in most laboratories to determine effectiveness against zoopathogenic organisms. For the most part these compounds have been found to be of little use to date because of low potency, toxicity to animals, and low stability.

Work in this laboratory has been directed toward the discovery, purification, and characterization of compounds occurring in plants which may be useful against phytopathogens causing destructive plant diseases. Such compounds should be less toxic to plant cells and might therefore be used advantageously for the control of plant pathogens.

This report deals with the discovery, isolation, and identification of 2-methoxy-1,4-naphthoquinone occurring in garden balsam (*Impatiens balsamina* L.). A short description of its antifungal activity is included. A semiquantitative assay procedure with an alcohol-tolerant strain of yeast as the susceptible test organism was used to facilitate the isolation. The utilization of this yeast permitted the testing of 100 per cent ethyl alcohol extracts of the plant material. This assay procedure is described.

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This naphthoquinone has been synthesized previously (21-23) but has not been reported as a natural product, although lawsone (2-hydroxy-1,4-naphthoquinone) was isolated from the leaves of *Lawsonia inermis* L (24-27).

The antibiotic compounds, fumigatin, javanicin, and spinulosin, are known to be methoxyquinones. Many other methoxyquinones have been found to possess marked antibiotic activity (28-30).

#### EXPERIMENTAL

*Microbiological Procedures*—The activity of crude juices obtained from *Impatiens balsamina* L was tested by the cylinder plate method described by Irving (31) with the Robbins agar medium (32). Media were adjusted to pH 7.0 with 0.05 N NaOH. Bacterial inoculum was produced in broth in 24 hours at 37°. Fungus spores were produced at 20° on Robbins' agar slants in 7 to 9 days. Yeast cells were propagated on Robbins' agar slants at 20° in 48 hours. On test plates bacteria grew only fairly well after 18 hours incubation, at which time zone readings were made. Fungus culture zone plates were stored at a constant temperature of 20° and, after incubation of 72 and 96 hours, readings were taken. The yeast was kept at 20° and cultures could be read after 48 hours incubation. Four penicylinders were placed on each Petri dish immediately after the agar had hardened and each filled with 0.3 ml. of liquid.

The red yeast *Rhodotorula glutinis* Harrison<sup>1</sup> served excellently as a test organism for alcohol solutions of crude juice extracts or for pure solutions of the crystalline material. In early work the organism was grown on agar slants and inoculum was produced by washing the slants with water. This 10 ml. suspension was added to 100 ml. of agar before flooding each test Petri dish with 3.0 ml. However, it was found more reliable and convenient to grow the yeast cells in Hansen's No. 2 liquid-aerated medium (33) for 4 days at room temperature. This cell suspension was then kept in the refrigerator at 8-10° and drawn on for uniform samples. Cells counted by means of a Levy chamber ran about 600 million per ml. When diluted with agar, this would mean that there were approximately  $143 \times 10^4$  cells per sq. cm.

LD<sub>50</sub> values and dosage-response curves were obtained by standard procedures (34) with *Monilinia (Sclerotinia) fructicola* as the test organism.

*Isolation Procedure*—After many trials the following method was developed. 675 gm. of the dried ground flowers of *Impatiens balsamina* L were extracted by stirring for 30 minutes with 4000 ml. of absolute ether. The slurry was filtered on a Büchner funnel and the cake extracted again

<sup>1</sup> The yeast *Rhodotorula glutinis* Harrison was identified by Professor F. M. Clark of the University of Illinois.

with 4000 ml. of ether. After filtration the filtrates, which were orange-yellow in color, were combined and concentrated to 1800 ml. This solution was run through a chromatograph column packed with 240 gm. of Brockmann's alumina. The filtrate which contained the active material was concentrated to dryness *in vacuo* and 900 ml. of petroleum ether (boiling point range, 35–45°) were added. After stirring for 20 minutes the material was filtered on a sintered glass funnel and the precipitate washed with petroleum ether and dried on the funnel. 507 mg. of a yellow crystalline material melting at 177–179° were obtained. This material was purified by repeated crystallization from absolute ethanol after treatment with norit and after filtering the hot solution. The highly purified material crystallized as light yellow needles and melted sharply at 183.5°.

*Chemical Properties*—The compound was found to be extremely soluble in chloroform and benzene, moderately soluble in alcohol and ether, and almost insoluble in water and petroleum ether. It was neutral to litmus and did not dissolve in aqueous acids. It dissolved in 3 N NaOH, giving a dark red solution. Acidification of this solution reprecipitated a yellow crystalline material.

No color was obtained with ferric chloride solution. A solution of the material in alcohol rapidly decolorized  $\text{KMnO}_4$  with the formation of  $\text{MnO}_2$ .

The material sublimes below the melting point and has a mild phenolic odor.

*Structure Proof*—A sodium fusion proved the absence of sulfur, nitrogen, and halogen.

Molecular weight by the Rast method was 168. This was later shown to be low, but explainable in the light of unreliability of this method, as applied to naphthoquinones.

Microanalysis<sup>2</sup> of a sample of the material melting at 183.5° gave the following values.

*Analysis*— $\text{C}_{11}\text{H}_8\text{O}_2$  (188.2)

Calculated, C 70.20, H 4.28; found, C 70.35, H 4.22

Since the analysis and properties of the material suggested either a methoxy- or a methylhydroxynaphthoquinone, a methoxyl determination was carried out with the following results.

*Analysis*— $\text{C}_{10}\text{H}_8\text{O}_2\text{-OCH}_3$  (188.2)

Calculated, methoxyl 16.49; found, 16.52

<sup>2</sup> All microanalyses were carried out by the Clark Microanalytical Laboratory, Urbana, Illinois.

In order to determine the location of the methoxy group a sample of the compound was first hydrolyzed to remove the methyl group (21), and then oxidized with  $\text{H}_2\text{O}_2$  as follows.

103 mg. of the demethylated material were dissolved in a minimum amount of 0.1 N NaOH by warming gently. The solution was cooled to room temperature and 5 ml. of superoxol added. After 2 hours a red crystalline precipitate separated but immediately redissolved upon the

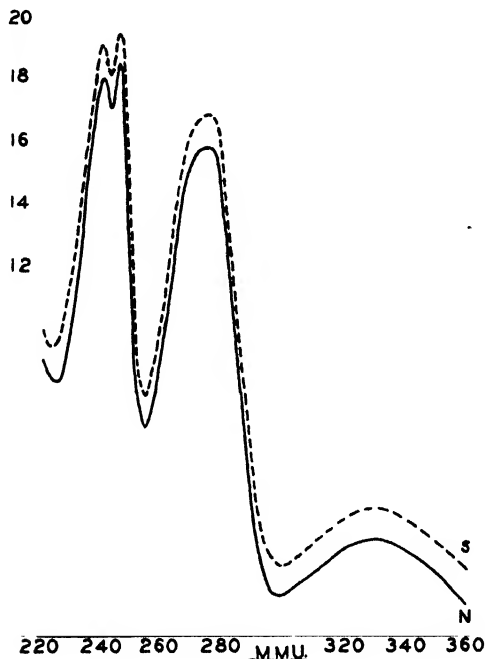


FIG. 1. Comparison of the ultraviolet light absorption curves of synthetic (Curve S) and natural (Curve N) 2-methoxy-1,4-naphthoquinones. The values for the synthetic compound are expressed as  $\epsilon \times 10^{-3} + 1000$  so that the curves will not be superimposed.

addition of a few drops of 12 N NaOH. After 5 hours standing at room temperature the solution became colorless. This was extracted ten times with an equal volume of ether to remove the excess  $\text{H}_2\text{O}_2$ . The solution was acidified by the addition of 3 ml. of 3 N HCl and extracted ten times with an equal volume of ether. The ether extract was concentrated to dryness *in vacuo* and the residue dissolved in 3 ml. of absolute alcohol and filtered. The alcohol was removed by evaporation and the solid material redissolved in a minimum of hot water. Norit was added and the solution filtered hot. Upon cooling a precipitate of colorless prismatic crystals

was obtained. This compound was recrystallized from hot water. The yield was 45 mg. The ground material melted at 191° with effervescence, resolidified at 108°, and remelted at 131°. There was no change in these values when the material was mixed with an equal quantity of phthalic acid. The analysis for this material was close to that for phthalic acid.

*Analysis*— $C_8H_6O_4$  (166.1)

Calculated, C 57.84, H 3.64; found, C 57.72, H 3.58

It was obvious, after identification of the oxidation product as phthalic acid, that the methoxy group was associated with the quinone ring of

TABLE I

*Zone Sizes Obtained against Various Organisms with Extract\* of Impatiens balsamina L*

Test organism	Zone
<i>Monilinia fructicola</i> 9962†.....	32.0
<i>Colletotrichum lindemuthianum</i> Vt-2..	31.3
<i>Aspergillus niger</i> 9642†.....	18.0
<i>Penicillium notatum</i> 9178†....	39.2
<i>Pythium debaryanum</i> 9998†.....	16.0
<i>Trichophyton mentagrophytes</i> 9533†.	0
<i>Ustilago avenae</i> 10056†.....	0
<i>Rhodotorula glutinis</i> Vt-3-1.....	35.6
"    "    Vt-3-1†.....	35.6
<i>Escherichia coli</i> 9637†.....	0
<i>Staphylococcus aureus</i> 9144†.....	37.7
"    "    6538P†.....	37.7
Florey strain (England).....	30.5

\* The extract was prepared as follows: 10 gm. of dried, ground flowers were extracted by stirring for  $\frac{1}{2}$  hour in 40 ml. of absolute alcohol. The slurry was filtered and the residue washed once with absolute alcohol. The filtrate was concentrated at 15° *in vacuo* to 20 ml. To this filtrate 70 ml. of distilled water were added and the solution again concentrated *in vacuo* to 30 ml. The solution was neutralized with NaOH and drawn through a Seitz filter. 1 ml. represents 0.33 gm. of dried material.

† American Type Culture Collection.

‡ Alcohol solution.

naphthoquinone, and it was suspected that the compound was 2-methoxy-1,4-naphthoquinone. This was confirmed by a spectrophotometric comparison with the pure synthetic compound (21). Solutions of both the natural and synthetic compounds were prepared by dissolving 10 mg. in 250 ml. of absolute alcohol which had been purified by treatment with norit followed by distillation. Higher dilutions were made when necessary



to maintain the sensitivity of the readings. The absorption readings were made on a Beckman model DU spectrophotometer. A comparison of the curves in Fig. 1 will show that the two compounds are identical. Also a mixed melting point (183.5°) showed no depression when compared to that of either compound.

*Antifungal Activity*—Table I lists the zone sizes obtained against several phytopathogenic fungi and against the assay yeast when tested as previously described. Both alcohol and water extracts were tested against the yeast. While the solubility in water is low, it was found that 10 mg. could be dissolved in 1000 ml. of distilled water if the material was finely ground and the suspension warmed to 70°.

Table II shows the effect of dilution upon the yeast assay zones.

TABLE II

*Effect of Dilution on Zone Sizes for Naturally Occurring 2-Methoxy-1,4-naphthoquinone with Rhodotorula glutinis As Test Organism*

Dilutions .....	1:0	1:2.5	1:5	1:7.5	1:10	1:15	1:25
	mm.	mm.	mm.	mm.	mm.	mm.	mm.
Zones in alcohol solution (25.1 mg. per 100 ml.)	37.3	35.1	30.6	22.1	19.6	15.6	8
Zones in water solution (9.6 mg. per 100 ml. supersaturated)	24.7	10.0	8	8	8	8	8

The LD<sub>50</sub> values (34) against *Monilinia (Sclerotinia) fruticola* for the natural and synthetic 2-methoxy-1,4-naphthoquinone were 0.00365 mg. per ml. and 0.00310 mg. per ml. respectively. The slopes of the dosage-response curves were 0.784 for the natural and 0.853 for the synthetic material. The methoxy compound was considerably more active than was lawsone.

Toxicity of a solution containing 10 mg. of the material in 1 liter of distilled water was tested against 3 week-old tomato and bean plants. No phytotoxicity was evident.

#### DISCUSSION

The isolation of 2-methoxy-1,4-naphthoquinone from a plant source, combined with the finding that this compound is non-toxic to tomato and bean plants, suggests that perhaps this material would have value as a fungicide. The high potency, as expressed in the LD<sub>50</sub> values and the slope of the dosage-response curve, also supports this idea. While its water solubility is low, it is certainly no lower than that of other common fungicides such as phygon and has the possible advantage of lower phytotoxicity.

The synthesis is relatively simple and cheaper methods could probably be developed.

The high antibiotic potency of methoxy-, as compared to hydroquinones, has been noted previously (30) and is confirmed in this instance.

#### SUMMARY

1. The existence of 2-methoxy-1,4-naphthoquinone in plant cells was established by its isolation from *Impatiens balsamina* L (garden balsam).
2. Details of its identification are presented.
3. The material is non-toxic to young tomato and bean plants in concentrations two to three times its LD<sub>50</sub> value.
4. Activities against several phytopathogenic organisms and others are listed.

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# THE LECITHIN, CEPHALIN, AND SPHINGOMYELIN CONTENT OF SERUM\*

## I. AS INDICATED BY THE CHOLINE-PHOSPHORUS AND NITROGEN-PHOSPHORUS RATIOS

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In 1944 Taurog, Entenman, and Chaikoff (13) reported that the ratio of choline to phosphorus in the acetone-insoluble lipides of human and dog plasma was close enough to 1.0 to justify the conclusion that cephalin was almost, if not entirely, absent. Although instances of the absence of cephalin from plasma had been reported before (1, 8, 14), most of the earlier work (cited in (13)) had indicated that some 20 to 40 per cent of the plasma phospholipides consisted of cephalins, or at least of non-choline-containing phospholipide. In spite of the wealth and consistency of the data presented by Taurog *et al.* (13) and later by Entenman and Chaikoff (3), a further and independent study of the problem seemed desirable. This was the primary object of the present work. However, the study was extended to the serum of beef, pig, and turkey, as well as to human and dog sera. Furthermore, the experiment was so planned as to obtain information on the sphingomyelin and lecithin, as well as on the cephalin, content of the various sera.

It so happened that at the same time Hack (9) had undertaken a somewhat similar study of the composition of human plasma phospholipides. His data show that in the fasting human subject the plasma phospholipides, on the average, consist of 79 per cent lecithin, 16 per cent sphingomyelin, and 5 per cent cephalin. In the individual subjects, however, cephalin was found to vary from 0 to 17 per cent of the total.

The general plan of the present work was to isolate the lipides from relatively large volumes of serum or plasma, to fractionate them into acetone-soluble and acetone-insoluble fractions, and to purify the latter in such a way as to insure that it contained only lipid nitrogen, phosphorus, and choline. Dialysis was chosen as the most satisfactory method of removing non-lipide impurities. The choline to phosphorus (C:P) ratio

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was taken to be a valid measure of the percentage of lecithin plus sphingomyelin. The difference between the N:P ratio and 1.0 was assumed to be a measure of the percentage of sphingomyelin in the total phospholipide. The validity of these two assumptions is dealt with in the accompanying paper (12).

#### EXPERIMENTAL

The lipides were isolated from three lots of beef sera, two of pig sera, two of pooled turkey sera, one of pooled dog sera, and one of pooled human sera.<sup>1</sup> All animals were either fasting or in a postabsorptive state. The volume of serum extracted ranged from 585 to 1170 ml. In addition, the lipides were extracted from the heparinized plasmas of four young men in a postabsorptive state.

*Extraction*—The details of the procedure employed were not strictly uniform throughout. Slight modifications were introduced with each successive experiment. Nevertheless, the general plan was the same and was as follows: The freshly drawn blood was collected in 250 ml. centrifuge bottles, chilled as soon as possible, and centrifuged in a refrigerated room. The pooled serum was recentrifuged. A small aliquot (5 or 10 ml.) was taken for determination of the lipide phosphorus content as a check on the completeness of the extraction of the main lot. This main lot was poured, with shaking, into 3 volumes of acetone. The mixture of serum and acetone was let stand at  $-33^{\circ}$  to  $-35^{\circ}$  and then filtered with suction at the same temperature. The object of this step was to precipitate most of the serum lipides, particularly the phospholipides, along with the proteins and to remove most of the serum water with the acetone. The filter cake was divided into suitable portions, thoroughly mixed in a Waring blender with an alcohol-ethyl ether mixture (3:1), let stand a short while with frequent shaking, and then filtered with suction. This alcohol-ether extraction was repeated. The filter cake was now blended with ethyl alcohol, poured into a sac made of filter paper, and extracted in a reflux extractor.<sup>2</sup> The filter paper and serum proteins were again mixed with alcohol and further extracted with hot alcohol. It was evident that the amount of lipide extracted by the two hot alcohol extractions made up a very small proportion of the whole.<sup>3</sup>

<sup>1</sup> The author is indebted to Dr. A. L. Fisher, the Connaught Laboratories, the University of Toronto, for the pooled human serum.

<sup>2</sup> This reflux extractor consisted of a Pyrex percolator connected to a 500 ml. Erlennmeyer flask and a 1 liter round bottomed flask as condenser.

<sup>3</sup> The completeness of the extraction procedure was demonstrated in two ways: In several cases the final alcohol extract was evaporated separately and the residue was extracted with petroleum ether-chloroform (2:1). The latter extract was

*Evaporation of Extracts*—The acetone extract, containing most of the serum water, was evaporated at about 40° under reduced pressure and in a gentle stream of nitrogen until water began to condense in the connecting tube. Little trouble from foaming was met with. The turbid concentrate was extracted twice with ethyl ether. The latter was put away in the refrigerator. The aqueous mother liquor now contained an insignificant amount of lipide, as shown by evaporation to complete dryness followed by extraction of the residue.

The alcohol-ether and alcohol extracts were evaporated as above, successive extracts being added as the earlier extracts became concentrated. The evaporation proceeded smoothly to dryness at 35–40° without any trouble from foaming. Finally the ether extract of the initial acetone extract was added and evaporated to dryness.

*Fractionation*—The light or deep yellow residue (depending on the species) was now repeatedly extracted with successive small portions of a petroleum ether-chloroform mixture (2:1) (PE-CHCl<sub>3</sub>). The insoluble portion was removed by centrifuging. The solution was aspirated into 100 ml. of acetone. The almost white precipitate that formed was packed by centrifuging and the clear acetone supernatant was poured off. The precipitate was redissolved in PE-CHCl<sub>3</sub>, centrifuged free of a small amount of insoluble material, and again precipitated with acetone. The acetone precipitation was repeated. The acetone-insoluble material was again dissolved in PE-CHCl<sub>3</sub> and put away in the refrigerator.

The three acetone supernatants were combined and evaporated almost to dryness in a stream of nitrogen under reduced pressure. The lipide residue was repeatedly extracted with warm acetone. The acetone extract was allowed to cool to room temperature and the resultant precipitate collected by centrifuging. The acetone-insoluble material was added to the earlier crop. To the clear acetone was now added 1 ml. of a saturated solution of magnesium chloride in ethyl alcohol. After vigorous shaking, the precipitate was centrifuged. The resultant clear acetone solution

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evaporated and weighed. The weight in all cases was very small. In several of the experiments, a small aliquot (5 or 10 ml.) of the serum was extracted exhaustively in an all-glass extraction outfit (Corning Glass No. 5200) with alcohol-ether (3:1) and alcohol. This extract was evaporated to dryness under reduced pressure and the lipides taken up in petroleum ether. The latter was concentrated and the lipides fractionated with acetone, with MgCl<sub>2</sub> to insure complete precipitation of the phospholipides. The phosphorus in the latter was determined colorimetrically. The yield of lipide phosphorus per 100 ml. of serum with the larger scale extraction in all cases was close to that of the small scale extraction, e.g. for pig serum 3.63 and 3.64 mg. respectively; for beef serum 2.88 and 2.92 mg. respectively, and for dog serum 12.64 and 11.89 mg. respectively.

was found by analysis to contain not more than a trace of phosphorus. It was saved for further study.

The precipitate of magnesium chloride and phospholipide was emulsified with water and dialyzed. The phospholipide emulsion was dried under reduced pressure. The resultant relatively small crop of phospholipide was either weighed and analyzed separately or combined with the main lot of acetone-insoluble material. An aliquot of this latter was now taken, dried, and analyzed for nitrogen, phosphorus, and choline.

*Purification*—It is well known from the observations of others, particularly MacLean (11), that the phospholipides isolated by acetone precipitation from the total lipid extracts of tissues contain quite large amounts of non-lipide impurities which are rich in nitrogen. The gross contamination of the phospholipides similarly obtained from blood plasma or serum has also been clearly demonstrated by the work of Folch and Van Slyke (7) and of Christensen (2). However, no data on the relative proportions of phospholipide and non-lipide impurities in the acetone-insoluble lipides from sera seem to have been published.

Such data have been obtained in this present work by dialyzing the emulsions of the crude acetone-insoluble lipides and determining the decrease in weight. Since the material was analyzed for nitrogen, phosphorus, and choline, both before and after dialysis, the change in these components as a result of the dialysis could be calculated. For dialysis, the acetone-insoluble lipid was freed from organic solvent under reduced pressure, emulsified with water, and transferred quantitatively to a Visking sausage casing. Dialysis was carried out at  $+4^{\circ}$  for 3 to 5 days in a rocking device against a steady flow of distilled water. The emulsion was kept stirred by marbles inside the sac. The lipid emulsion was now dried under reduced pressure at a low temperature. The lipid residue dissolved in  $\text{PE-CHCl}_3$  to form a clear, very faintly yellow solution.

Analysis of these dialyzed serum phospholipides showed that the absolute content of N, P, and choline was below that calculated for a mixture of lecithin, cephalin, and sphingomyelin. After considerable study it was discovered that, in spite of the earlier repeated precipitations with acetone, the material contained an appreciable amount of free fatty acids together with a small amount of neutral lipid that gave a strong reaction for fatty aldehyde with Schiff's reagent (5). These could be removed by precipitation of the dialyzed phospholipide with acetone.

Accordingly the following procedure was adopted: The bulk of the phospholipide was precipitated from the concentrated  $\text{PE-CHCl}_3$  solution with acetone. The precipitate was washed once with acetone and then taken up in  $\text{PE-CHCl}_3$  and stored in the refrigerator. To the clear supernatant acetone, magnesium chloride in alcohol was added and the resultant precipitate centrifuged. This further crop of phospholipide was emulsified

with water, dialyzed free of magnesium chloride, dried, and combined with the bulk of the phospholipide.

*Analyses*—Aliquots of the PE-CHCl<sub>3</sub> solutions were freed of solvent in a stream of N<sub>2</sub> and dried over P<sub>2</sub>O<sub>5</sub>. This weighed sample was redissolved in chloroform. A suitable aliquot (40 to 100 mg.) was pipetted for the determination of nitrogen and phosphorus and another aliquot for choline determination.

For *nitrogen* and *phosphorus* analysis, the sample was digested with 3 ml. of fuming sulfuric acid and 1 gm. of catalyst (copper sulfate 1 part, potassium sulfate 9 parts) for at least 4 hours on an electric heater. A small piece of sintered glass served as an efficient antibumping agent. The digest was dissolved in 25 ml. of distilled water. A 10 ml. aliquot was distilled in a micro-Kjeldahl apparatus<sup>4</sup> to determine the nitrogen content,

TABLE I  
*Replicate Analyses on Egg Lecithin*

Date	N	P	Choline	N:P	C:P
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Jan. 25	1.80	3.91		1.02	
“ 27	1.75	3.80	16.95, 16.81 16.90, 16.90	1.02	0.99
Feb. 3	1.80	3.96		1.01	
“ 18	1.83	3.99	17.35	1.01	0.97
Mar. 13	1.79	3.95	17.18	1.00	0.97
Apr. 14	1.76	3.95	17.19, 17.00 16.93, 16.82	0.99	0.96
“ 22					
Average.....	1.79	3.93	17.00	1.01	0.97

boric acid being used to absorb the ammonia. A 2 ml. aliquot, diluted to 25 or 50 ml., was used to determine the phosphorus content by the method of Fiske and Subbarow (6).

*Choline* was determined by the method of Entenman and Chaikoff (3), as prescribed by the authors, except that the choline reineckate was dissolved in 10 or 25 ml. of acetone in volumetric flasks.

All colorimetric measurements were made with a Coleman No. 11 spectrophotometer.

The three analytical methods were checked repeatedly by analyzing known solutions and also by the analysis of a purified sample of egg yolk lecithin.

Since the latter results offer the best basis for assessing the significance of the data obtained on the sera phospholipides, they are set forth in complete form in Table I. These data need little discussion. Inspection

<sup>4</sup> Scientific Glass Apparatus Company No. M-3065.



shows that the range in the C:P ratio, to which particular importance will be attached, is of the order of 0.03. Since the purified egg lecithin contained no detectable amino N, the correct C:P ratio is presumably 1.00. The data indicate that the choline values tended to be somewhat too low. It has been our experience that one of the larger sources of variation in the *absolute* percentages of nitrogen, phosphorus, and choline is the difficulty in getting the true dry weight of the sample, due to the tendency of phospholipide to retain water or alcohol, on the one hand, and to oxidize on the other. The procedure used in this study was so chosen as to minimize factors that could reduce the accuracy of the N:P and C:P ratios.

TABLE II  
*Composition of Acetone-Insoluble Lipides*

Serum	N	P	Choline	N:P	C:P
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Pooled human.....	2.40	2.70	12.20	1.97	1.00
Human I*.....	1.98	2.51	10.84	1.75	0.92
“ II*.....	2.39	3.09		1.71	
“ III*.....	2.29	3.08	13.04	1.65	0.92
“ IV*.....	2.22	3.16		1.56	
Beef I.....	2.49	2.47	11.40	2.23	1.03
“ IIa.....	2.65	2.95	12.84	1.99	0.97
“ IIb.....	2.67	2.91	12.80	2.03	0.98
Pig I.....	2.84	2.57	12.45	2.45	1.08
“ II.....	2.66	2.22	11.29	2.66	1.13
Dog.....	3.40	3.17	14.36	2.38	1.01
Turkey I†.....	1.53	2.34	9.50	1.45	0.90
“ II†.....	1.71	2.49	9.20	1.52	0.82

\* In these cases plasma was used.

† All material insoluble in cold ether was removed.

### *Results*

The data on the nitrogen, phosphorus, and choline contents and on the N:P and C:P ratios of each of thirteen samples of the acetone-insoluble lipides from serum or plasma are given in Table II. Attention is first directed to the experiment in which two 1 liter portions of the same lot of beef serum (IIa and b) were carried in parallel through the entire procedure. The closeness of the analytical data supplements those presented in Table I on egg lecithin in demonstrating the satisfactory precision of the methods used.

From the point of view of the primary purpose of the study, the C:P ratios given in the last column in Table II are of the most interest. It may be seen that in the case of the pooled human, beef, and dog sera, the C:P

ratios are, within the error of the methods, equal to 1.00. These data thus can be regarded as a complete confirmation of the results of Taurog *et al.* (13). On the other hand, the C:P ratios on the acetone-insoluble lipides from human plasma and especially from turkey serum were definitely below 1.00, indicating the presence of a significant proportion of cephalin. In the case of the two pig sera, the C:P ratios were significantly above 1.00.

The magnitude of the nitrogen values and of the N:P ratios for the crude acetone-insoluble lipides from serum shows very strikingly how grossly contaminated these lipides are by nitrogen-rich impurities. However, this is brought out still more clearly by the data in Table III which show the percentage loss in weight, nitrogen, phosphorus, and choline on dialysis of twelve samples of serum acetone-insoluble lipides. It may be

TABLE III  
*Per Cent Loss by Dialysis of Crude Acetone-Insoluble Lipides*

Serum	Weight	N	P	Choline
Pooled human.....	20.5	27.0	1.5	10.5
Human I.....	36.0	33.4	3.8	
“ II.....	21.8	29.2	0	
“ III.....	26.0	31.2	7.3	
“ IV.....	21.5	27.0	2.4	
Beef I.....	37.6	46.5	10.9	11.8
“ IIa.....	28.7	39.6	8.1	12.1
Pig I.....	35.2	52.8	7.8	19.5
“ II.....	40.1	51.9	1.4	12.2
Dog.....	25.7	57.4	14.2	16.7
Turkey I.....	24.5	32.0	0	15.4
“ II.....	24.3	40.9	12.4	5.1

seen that from 21 to 40 per cent of the crude material diffused through the membrane and was, therefore, presumably non-lipide in nature. From 27 to 57 per cent of the nitrogen was diffusible. The loss in phosphorus was in some cases insignificant, in others quite appreciable. In all cases choline was lost during dialysis in amounts ranging from 5 to 20 per cent of the total.

In assessing the meaning of these losses, particularly of choline, one naturally wonders whether or not appreciable hydrolysis of phospholipide could occur during the 3 to 5 day dialysis at +4°. To settle this question a sample of almost pure egg lecithin was dialyzed for 5 days and then put through the standard procedure. Of the initial material 95.6 per cent was recovered by drying the emulsion left in the sac. The percentages of N, P, and choline on the non-dialyzed and dialyzed lecithin were as follows:

1.79, 1.79; 3.95, 3.93; 17.18, 17.07. There were, therefore, only a slight loss in weight and no detectable change in the composition of lecithin as a result of the dialysis. The very considerable and consistent loss of weight and of choline, and the less striking loss of phosphorus during the dialysis of the crude acetone-insoluble lipides from sera, presumably, therefore, are due to the removal of non-lipide contaminants.<sup>5</sup>

The N, P, and choline contents and the N:P and C:P ratios of the eleven samples of serum or plasma phospholipides after purification by dialysis and reprecipitation by acetone are shown in Table IV.

TABLE IV  
*Composition of Acetone-Insoluble Lipides after Dialysis and  
Reprecipitation by Acetone*

Serum	N	P	Choline	N:P	C:P
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Pooled human*	2.28	3.64	15.03	1.39	0.92
Human I†	2.06	3.78	15.87	1.21	0.93
“ II†	2.17	3.97	16.46	1.21	0.92
“ III†	2.13	3.86	16.22	1.22	0.94
“ IV†	2.07	3.93	16.29	1.17	0.92
Beef I*	2.14	3.80	16.57	1.25	0.97
“ II (a + b)	2.37	3.99	17.16	1.32	0.96
Pig I + II*	2.09	3.80	(16.57)‡	1.22	(0.97)‡
Dog*	2.01	3.88	16.40	1.15	0.94
Turkey I§	1.63	3.64	12.98	0.99	0.79
“ II§	1.61	3.40	11.93	1.05	0.78

\* The portion insoluble in cold petroleum ether had been removed.

† In these cases plasma was used.

‡ These analyses apply to the dialyzed phospholipide before precipitation with acetone.

§ All ether-insoluble material had been removed.

It is clear that in all cases except the two lots of turkey sera<sup>6</sup> the phospholipides contained not more than about 3 to 8 per cent of cephalin. The turkey serum phospholipides contained slightly over 20 per cent cephalin.

As stated above, it was tentatively assumed that the N:P ratios of the purified phospholipides could be taken as a measure of the sphingomyelin content. On this basis, the data in Table IV indicate that sphingomyelin

<sup>5</sup> It is of course uncertain whether diffusable choline is present in the crude acetone-insoluble lipides or whether some other contaminant gives an insoluble reineckate and thus contributes to the apparent choline present.

<sup>6</sup> Dr. I. L. Chaikoff has informed the author that the C:P ratios of chicken plasma phospholipides have been found to range between 0.89 and 0.92.

made up from 15 per cent of the phospholipides in dog serum to 32 per cent in beef serum. The considerably higher value for the N:P ratio of the phospholipides from the pooled human serum than from the four samples of human plasma calls for comment. The explanation is not at all clear. It may, however, be the result of some cleavage of phospholipide during the storage of the pooled serum and that this hydrolysis affected the lecithin more than the sphingomyelin. It is noteworthy that the yield of phospholipide from the pooled serum was lower than from the fresh plasmas. The low values for the N:P ratios of the phospholipides of the turkey sera were due to the fact that the ether-insoluble portion has been removed and analyzed separately.

A comparison of the *absolute* values for the N, P, and choline contents of the various samples of serum or plasma phospholipides after purifica-

TABLE V  
*Amount and Composition of Phospholipide in Serum or Plasma*

Source	Total lipide P	Total phos- pholipide	Lecithin	Cephalin	Sphingo- myelin
	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.
Pooled human.....	5.11	140	74	11	55
Human Plasma I.....	7.87	208	150	14	44
“ “ II.....	6.57	165	117	13	35
“ “ III.....	5.92	154	111	9	34
“ “ IV.....	10.07	256	192	20	44
Beef Serum I.....	3.16	83	60	2	21
“ “ II.....	2.98	75	48	3	24
Pig Serum.....	3.63	96	72	3	21
Dog “.....	14.18	365	288	22	55

tion with those required of a mixture of lecithin, cephalin, and sphingomyelin shows reasonably satisfactory agreement. The agreement in the phosphorus values is especially significant, since it indicates that cerebroside is not an important component of the acetone-insoluble lipides and thus lends support to the use of the N:P ratio as a measure of the sphingomyelin content. This point is more directly dealt with in the accompanying paper (12).

#### DISCUSSION

It is very evident that the data presented in this paper strongly support the conclusion first reached by Taurog *et al.* (13), and later confirmed by Entenman and Chaikoff (3) and by Hack (9), that the phospholipides in human and in dog plasma are mainly choline-containing, and thus that

cephalin is at most a minor component. The same has now been shown to hold for the serum phospholipides of the beef and of the pig. On the other hand, it is clear that this small proportion of cephalin cannot be regarded as a fixed characteristic of plasma phospholipide, since that of turkey serum contains about 20 per cent of cephalin. In view of the evidence that phospholipide undergoes a rapid exchange between plasma and the liver (4, 10), the disparity in the relative proportions of lecithin and cephalin in plasma and in liver creates a very interesting and undoubtedly important problem.

The total amount of purified phospholipide expressed in terms of phosphorus and of the three components, lecithin, cephalin, and sphingomyelin, as calculated from the C:P and N:P ratios, in the serum or plasma of man, beef, pig, and dog is given in Table V.

#### SUMMARY

The acetone-insoluble lipides from fairly large volumes of human, beef, dog, pig, and turkey sera, and from four samples of human plasma, were isolated and analyzed for N, P, and choline. They were then dialyzed to remove non-lipide impurities, reprecipitated with acetone, and again analyzed.

The choline to phosphorus ratios for the purified acetone-insoluble lipides indicate that cephalin makes up about 20 per cent of the phospholipides in turkey serum and about 3 to 8 per cent of the phospholipides in human plasma and in the serum of beef, dog, and pig.

The N:P ratios for the purified phospholipides indicate that sphingomyelin makes up 15 to 32 per cent of the total (discounting the figure of 39 per cent obtained from pooled human sera that had been stored for some time).

The crude acetone-insoluble lipides were found to contain 21 to 40 per cent of diffusible, non-lipide impurities which were rich in nitrogen and apparently included some choline in all cases.

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## THE LECITHIN, CEPHALIN, AND SPHINGOMYELIN CONTENT OF SERUM\*

### II. AS INDICATED BY THE AMINO NITROGEN AND NON-DIFFUSIBLE PHOSPHORUS CONTENT AFTER DIFFERENTIAL HYDROLYSIS OF THE ACETONE-INSOLUBLE LIPIDES

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In an accompanying paper (4) the author presented data on the choline to phosphorus (C:P) and nitrogen to phosphorus (N:P) ratios of the serum phospholipides of a number of animals before and after purification by dialysis. Since the C:P ratios in all instances were somewhat below 1.00, it was concluded that cephalin was probably present in all the sera studied, although it made up, as a rule, only about 3 to 8 per cent of the total phospholipide. Only in the case of turkey serum was the C:P ratio indicative of a quite substantial proportion of cephalin, about 20 per cent of the total.

The presence of cephalin is certainly the most reasonable interpretation of a C:P ratio less than 1.00. Nevertheless, other possible explanations had to be considered. The most obvious and most annoying possibility is that the method used for the determination of choline was giving results that were somewhat too low. Indeed, analysis of a highly purified sample of egg lecithin gave C:P ratios ranging from 0.96 to 0.99 whereas no amino nitrogen could be detected. Another possibility is that the serum or plasma contains a small amount of nitrogen-free phospholipides such as phosphatidic acid for example. It seemed, therefore, advisable to verify the presence of cephalin by the direct determination of the amino nitrogen content of the serum phospholipides.

The N:P ratios of the purified serum or plasma phospholipides were found to range between 1.15 and 1.32. On the assumptions that all the non-diffusible nitrogen was actually lipide-bound nitrogen and that cerebrosides were at most a minor component of the serum lipides, these N:P ratios were taken as a measure of the sphingomyelin content of the phospholipides. It was felt that the validity of these assumptions should be verified by an independent and more direct determination of sphingomyelin.

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In order to meet both objectives, the method of Schmidt *et al.* (3) was adapted so that, after alkaline hydrolysis of the lecithins and cephalins, the water-soluble glycerophosphate, choline, and ethanolamine (or serine) could be separated from the non-hydrolyzed sphingomyelin by either solvent fractionation or by dialysis. Thus, the amino nitrogen content of the water-soluble or diffusible fraction was taken as a measure of the cephalin content, whereas the phosphorus content of the water-insoluble or non-diffusible fraction was taken as a measure of the sphingomyelin content.

#### EXPERIMENTAL

Portions (133 to 300 mg.) of the serum phospholipides, purified by dialysis followed by reprecipitation with acetone (4), were dissolved or suspended in 1 ml. of ethyl alcohol and then mixed with 20 ml. of *N* potassium hydroxide in a centrifuge tube. The tubes were rotated on a wheel at 37° for 22 (first experiment) or 34 (second experiment) hours. Concentrated hydrochloric acid was then added until the contents were just acid to Congo red paper.

In the first experiment, an attempt was made to fractionate the hydrolyzed mixture into water-soluble and water-insoluble fractions by the use of solvents. The contents of each tube were extracted repeatedly with a mixture of petroleum ether and chloroform (2:1). At first a heavy interfacial layer formed after the tubes were centrifuged. The PE-CHCl<sub>3</sub> extract was evaporated to dryness under reduced pressure. The residue of fatty acids and unhydrolyzed lipide was now extracted with acetone several times. The acetone-insoluble material was dried and analyzed for nitrogen and phosphorus.

The aqueous hydrolysate was evaporated to dryness and extracted with ethyl alcohol in order to get rid of the large amount of potassium chloride. An aliquot of the alcohol extract was taken for phosphorus determination. The remainder was evaporated to dryness, taken up in 2.5 ml. of water, and used for the amino nitrogen determination (5).

In the second experiment only petroleum ether was used for the extraction of the hydrolyzed mixture. Each tube, after being centrifuged, contained three sharply defined layers: the lower aqueous layer, an interfacial layer of gel-like consistency, and a clear upper petroleum ether layer. This petroleum ether extract was evaporated and the residue fractionated into acetone-soluble and acetone-insoluble fractions, magnesium chloride being used to make the fractionation more complete.

The resultant acetone-insoluble material was combined with the aqueous layer and the interfacial material and transferred to a small dialysis sac. This sac was enclosed in a large glass-stoppered tube, which was then filled

with water (20 to 30 ml.). The tube was allowed to stand for several hours with repeated inversion. The water outside the sac was then poured off and a fresh lot of water added. This process was repeated eleven times. The resultant solution of the diffusible, water-soluble components of the hydrolysis mixture was evaporated to dryness and the residue extracted with alcohol. After removal of an aliquot for P determination, the remainder was used for amino nitrogen determination as above.

The non-diffusible material in each sac was further dialyzed against flowing distilled water and then freed of water by evaporation under reduced pressure. The residue was dissolved in chloroform and an aliquot taken for N and P determinations.

TABLE I

*Amino Nitrogen Determinations after Hydrolysis of Serum or Plasma Phospholipides*

Source	Amino N	NH <sub>2</sub> -N:P	C:P*
	<i>per cent</i>		
Human Plasma I	0.10	0.06	0.93
" " II	0.07	0.04	0.92
" " III	0.06	0.03	0.94
" " IV	0.06	0.03	0.92
Beef Serum I	0.00		0.97
" " II	0.00		0.96
Pig " I + II	0.07	0.04	0.97
Dog " "	0.00		0.94
Turkey Serum I + II	0.25, 0.26	0.16	0.79

\* From the accompanying paper (4).

### Results

*Amino Nitrogen Content of Hydrolysate*—The yields of amino nitrogen expressed as percentages of the serum phospholipides hydrolyzed are given in the second column of Table I. It will be seen that in three instances, the two beef sera and dog serum, no amino nitrogen was found. The turkey serum phospholipides yielded quite substantial amounts of amino nitrogen, with good agreement between the duplicate determinations. The human plasma and pig serum phospholipides yielded small but quite significant amounts of amino nitrogen.

In Table I the amino nitrogen content of the mixed phospholipides is also expressed as the NH<sub>2</sub>N:P ratio. The C:P ratios are given as in the accompanying paper (4). If all the lipide P of serum is accounted for as lecithin, sphingomyelin, and a little cephalin, the NH<sub>2</sub>N:P and C:P ratios should add up to 1.00. Actually, the precision of the methods was not such as to expect such a result. In eight out of the nine cases, the sum of

these two ratios fell within the range, 0.95 to 1.01. These results thus offer definite evidence that cephalin is a real, though very minor, component of plasma phospholipides. The only discrepancy is in the case of the dog serum phospholipides, which gave no amino nitrogen, although the C:P ratio was 0.94.

*Phosphorus Distribution after Differential Hydrolysis of Serum Phospholipides*—The data obtained in the first experiment involving differential hydrolysis of the phospholipides with alkali are given in Table II. It will be seen that 13 to 31 per cent of the total lipid phosphorus was recovered in the water-insoluble, acetone-insoluble fraction after hydrolysis. Presumably this fraction contains all the phosphorus present in the mixture as sphingomyelin. Now, as pointed out above, if the mixed acetone-insoluble lipides of serum consist only of lecithin, a little cephalin, and sphingomyelin, then  $[(N:P) - 1] \times 100$  should be a measure of the percentage

TABLE II  
*Distribution of Phosphorus after Hydrolysis of Serum Phospholipides*

Serum	Intact phospholipide,* [(N:P) - 1] × 100	Water- and acetone-insoluble P	Hydrolyzed phospholipide	
			Water-soluble P	Total P recovered
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Beef I	25	18.9	71.2	90.1
" II	32	31.0	54.5	85.5
Dog	15	13.0	70.4	83.5
Pig	22	15.3	68.2	83.5

\* From the accompanying paper (4).

of sphingomyelin in the mixture. The values for sphingomyelin given by these two independent methods should agree. If, however, a considerable amount of cerebroside were present as well as sphingomyelin, the value estimated from the N:P ratio would be the higher. Both sets of values are given in Table II. It may be seen that in two cases the results obtained by the two methods agree reasonably well; in the other two cases there is a considerable discrepancy between them, the value obtained by the method of differential hydrolysis being considerably lower than the other.

It will be seen that the sum of the water-insoluble, acetone-insoluble P and the water-soluble phosphorus after hydrolysis was very significantly below 100 per cent of that in the unhydrolyzed phospholipide. The reason for the loss is unknown. In the hope of improving the recovery of phosphorus, in the second experiment dialysis was used as a means of fractionating the phosphorus-containing compounds, as described in the ex-

perimental section. The results obtained in this second experiment are given in Table III.

In this second experiment the values for the percentage of sphingomyelin as given by the non-diffusible phosphorus are almost the same as those given by the expression  $[(N:P) - 1] \times 100$ . This fact would seem to indicate that cerebrosides are not present in significant amounts. However, since material was still available, it was decided to investigate the question more directly. The residual portions of the non-diffusible fraction of the hydrolyzed acetone-insoluble lipides of human plasma were combined and analyzed for galactose by the method of Brand and Sperry (1).

This material was found to contain 2.2 per cent galactose, which is equivalent to 10 per cent cerebroside. Thus, this single determination suggests that cerebrosides make up only about 2 per cent of the total acetone-insoluble lipides of human plasma. Since this proportion is small

TABLE III

*Distribution of Phosphorus after Hydrolysis of Human Plasma Phospholipides*

Human plasma No.	Intact* phospholipide $[(N:P) - 1] \times 100$	Hydrolyzed phospholipide		Total P recovered
		Non-diffusible P	Diffusible P	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
I	21	20.8	71.9	92.7
II	21	23.1	69.4	92.5
III	22	23.1	69.1	92.2
IV	17	19.9	74.5	94.4

\* From the accompanying paper (4).

as compared with the sphingomyelin content, it is evident that the latter is largely responsible for the N:P ratio in excess of 1.0.

#### DISCUSSION

The demonstration of small but significant amounts of amino nitrogen in the water-soluble hydrolysate of the phospholipides from each of the four samples of human plasma and from pig and turkey sera would seem to prove beyond any doubt that cephalin actually is a component of the plasma phospholipides in these animals. These direct determinations thus confirm the conclusion drawn from the C:P ratios. On the other hand, the failure to detect any amino nitrogen at all in the phospholipides from beef and dog sera shows that the C:P ratio is not always a reliable indication of the presence of cephalin.

The values for the sphingomyelin content of human plasma, as calculated from the percentage of non-diffusible phosphorus after alkaline hydrolysis

of the mixed phospholipides, agree reasonably well with the data obtained by Schmidt *et al.* (3) and by Hack (2) by essentially the same method. The conditions chosen for differential hydrolysis (34 hours at 37° in N KOH) evidently caused no splitting of the amide linkage or of the phosphoric ester linkage in the sphingomyelin. However, some splitting of the choline-phosphoric acid ester linkage evidently did occur, since the N:P ratios of the non-diffusible material were considerably below the value of 2.0 required for sphingomyelin. Conversely the N:P ratios of the diffusible fraction were considerably above 1.0. It is clear, therefore, that time of hydrolysis is a very important factor in Hack's adaptation of the Schmidt procedure for the determination of the lecithin, cephalin, and sphingomyelin content of a mixture.

#### SUMMARY

The acetone-insoluble lipides of human plasma, and of beef, dog, pig, and turkey sera, after purification by dialysis, were hydrolyzed with 1 N KOH. Water-soluble amino nitrogen was found in sufficient amounts to indicate that cephalin makes up about 3 to 6 per cent of the total phospholipide in human plasma and pig serum but about 16 per cent of that in turkey serum. On the other hand, no cephalin was found in beef or dog serum.

The phosphorus-containing compounds, after hydrolysis, were fractionated into diffusible and non-diffusible fractions. The latter, the sphingomyelin phosphorus, was found to make up 15 to 32 per cent of the total phosphorus. These values for sphingomyelin are in quite good agreement with those calculated from the N:P ratio of the mixed acetone-insoluble lipides.

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## A DETAILED PROCEDURE FOR THE PREPARATION OF HIGHLY PURIFIED ADENOSINE TRIPHOSPHATE\*

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A number of procedures for the preparation of adenosine triphosphate (ATP) have appeared in the literature (1-11) and methods have been described briefly for increasing the purity of partially purified ATP (10, 12-14). These methods yield ATP in various states of purity, but either they are tedious or do not yield pure product, or are described in insufficient detail to permit a biochemist not expert in isolating phosphorylated products to obtain the ATP in the reported yields without difficulty and loss of time. In some cases too few analytical data are recorded to define the purity of the product.

The following method for preparing ATP is presented in sufficient detail to be easily repeated by a student in an advanced biochemistry course, and yields ATP of a high degree of purity and in reasonably good yield. The method has been checked by a number of workers in this laboratory, including several graduate students, and by one worker in another laboratory.<sup>1</sup> 2 to 3 days of intermittent work are required to obtain the product from one or two rabbits. The method can also be applied to properly washed yeast with one very slight modification.

There are no new principles involved in the method, which is based upon procedures originally employed by Lohmann and Schuster (5) and by Kerr (6). The success of the method probably depends upon the order in which the precipitations with mercury and barium are carried out and in the exact control of experimental conditions at each step.

The ATP prepared as outlined above has been tested for biological activity by using washed yeast cells. In the presence of glucose the ATP is converted to adenylic acid but no inorganic phosphate appears; in the absence of glucose the ATP is hydrolyzed to adenylic acid and free inorganic phosphate, both of which can be recovered quantitatively in the

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medium. In these experiments the ATP prepared from rabbit or yeast behaves in the same manner. In addition, rabbit ATP has been used for the preparation of adenosine diphosphate (ADP) by a modification of the method of Lohmann (15) with lobster muscle, yielding ADP in a high state of purity.

#### EXPERIMENTAL

##### *Preparation of Animal*

*Without Curare*—The preparation of the animal is one of the most important steps in the procedure for obtaining ATP. If this is done carelessly, the yield may be very low. In most of our experiments we have anesthetized the rabbit with nembutal, given very slowly by ear vein. When complete anesthesia is obtained, one carotid artery is isolated and a longitudinal slit is made in it with a sharp scalpel. The animal is then allowed to bleed to death. In repeated experiments it has been found that this procedure leads to at least as good yields of ATP as does the method of DuBois, Albaum, and Potter (8) with magnesium sulfate anesthesia. However, the latter method may be more useful to an experimenter not trained in physiological techniques.

As soon as the animal is dead, it is quickly skinned. If nembutal alone or magnesium sulfate has been used for anesthetic, the skin is cut off at the neck, care being taken not to break the back or neck of the animal. The viscera are quickly removed and the body is placed in a bucket of ice water containing a large amount of ice. When the temperature of the carcass is in the neighborhood of 5°, the muscles can be cut out without any twitching whatsoever. The hind leg and back muscles and, if desired, the fore leg and pectoral muscles are dissected as quickly as possible and are placed in a beaker containing ice and water. It is of advantage to carry out the work on the carcass in a cold room if possible. It is also of some advantage to place the muscles in liquid air instead of an ice-water mixture, so that subsequently they can be broken up into small pieces with a hammer or in a meat grinder, in order to minimize slowing of the Waring blender in subsequent steps because of the presence of long strands of connective tissue.

*With Curare*—Nembutal is administered by vein until deep anesthesia is produced, and the animal is allowed to remain in the anesthetized state for 20 minutes. Then 15 units of intercostin (Squibb) are rapidly injected intravenously. The respiration of the animal stops immediately. After 1 to 2 minutes the animal is decapitated (or allowed to bleed from a previously isolated carotid artery) and as much blood as possible is drained. The abdominal and intercostal muscles can be used as well as the leg and back muscles.

The use of curare probably permits more latitude in preparation of the animal and permits the use of more of the muscle mass. It is doubtful whether the yield of ATP per kilo of muscle is materially increased over the yield that can be obtained if the procedure without curare is carried out with sufficient care.

After the animal is dead, all subsequent operations described above should be carried out as rapidly as possible, regardless of what anesthetic has been used. It is better not to obtain all the available muscle than to spend more than half an hour in dissecting small pieces. A 4 kilo rabbit should yield about 1 kilo of muscle.

### *Preparation of Adenosine Triphosphate from Rabbit Muscle*

1. Cut the muscle into tiny bits with scissors, or else run it once through an ice-cold, hand-operated meat grinder. Mix 100 gm. portions of the muscle in a Waring blender with 200 ml. portions of ice-cold 8 per cent trichloroacetic acid. About 2 minutes of mixing in the blender will be required for each portion. Small amounts of crushed ice should be added to the blender to keep the extraction mixture at a low temperature. Pour the mixture from the blender into a double layer of cheese-cloth, and then make a bag of the cheese-cloth and squeeze out the liquid, working the mass of muscle with the hands until practically no liquid remains in the bag. Rubber gloves should be worn, if possible, to avoid irritation of the hands by the trichloroacetic acid. This work should be carried out in a cold room to insure maximal yields.

When all of the muscle has been extracted with 8 per cent trichloroacetic acid, weigh the residue from the cheese-cloth bags and reextract by mixing 200 gm. portions of the residue with 400 cc. of 5 per cent trichloroacetic acid in the blender for 2 minutes, with the addition of small amounts of crushed ice, as before.

Squeeze the second extract of the muscle through cheese-cloth bags as before, and combine the first and second extracts. Filter the combined extracts through fluted filter paper in the cold room or ice box. At this stage, and at all subsequent steps in which the solution of ATP is acid, it must be kept as close to 0° as possible.

2. Neutralize the filtrate from step (1) with 10 per cent NaOH to pH 7.2 to 7.5 (using bromothymol blue and phenol red indicator solutions on a spot plate as a control). About 300 ml. of 10 per cent NaOH may be required for the muscle from one good sized rabbit.

3. Add glacial acetic acid to the neutralized solution from step (2) in the cold with stirring until the concentration of acetic acid in the solution is 0.2 per cent. Next add slowly and with stirring 5 ml. of 20 per cent mercuric acetate dissolved in 2 per cent acetic acid for each 100 ml. of the original



trichloroacetic acid filtrate. Allow the precipitate to settle in the ice chest, decant, and centrifuge. Discard the supernatant. Wash the precipitate once with 0.5 per cent mercuric acetate (made by diluting the 20 per cent mercuric acetate in 2 per cent acetic acid 1:40 with distilled water). An amount of 0.5 per cent mercuric acetate equal to about 2 or 3 times the estimated volumes of the nucleotide precipitate should be used in this washing.

4. Suspend the washed nucleotide precipitate in enough water to fill one large centrifuge tube (about 200 ml.) and pass in  $H_2S$  for 1 hour, cooling the tube in ice-water. Centrifuge the precipitate of  $HgS$  and save the supernatant.

Resuspend the precipitate of  $HgS$  in about 100 ml. of water and treat again with  $H_2S$  for 15 minutes, cooling the tube again in ice-water. Centrifuge the  $HgS$  (to be discarded) and add the supernatant to the supernatant from the first treatment of the nucleotide suspension with  $H_2S$ . Now pass a rapid stream of air into the combined supernatants (which should be allowed to warm up to room temperature) until the solution is practically free from  $H_2S$ , judging by the odor.

5. Adjust the pH of the aerated solution to 7.0, using NaOH with bromothymol blue as indicator. About 10 ml. of 10 per cent NaOH may be required for the material from one rabbit. Then dilute to 500 ml. with distilled water and add 1 ml. of 2 M barium acetate per 100 gm. of muscle used. Let the precipitate settle (in the ice chest) and then decant and filter. The supernatant, which contains coenzymes, nucleotides, sugar phosphates, etc., can be discarded or saved for precipitation of the water-soluble, alcohol-insoluble barium salts of some of these materials if desired (with about 4 volumes of alcohol per volume of supernatant). The precipitate of water-insoluble barium nucleotides is then washed twice with about 1 to 2 volumes of ice-water.

6. The washed barium nucleotide precipitate is now suspended in about 300 ml. of water and is dissolved by adding the minimal amount of glacial acetic acid slowly and with stirring. The pH should be between 3 and 3.5 when solution is complete. (10 to 15 ml. of glacial acetic acid may be required for the material from one rabbit.) A small amount of undissolved precipitate is centrifuged off and discarded.

7. Now dilute the dissolved barium nucleotides to 500 ml. and add 0.5 ml. of 20 per cent mercuric acetate in 2 per cent acetic acid per 100 ml. of the original trichloroacetic acid filtrate, slowly and with stirring. Allow the precipitated mercuric nucleotides to settle (in the ice box) and decant and centrifuge. Wash the precipitated nucleotides once with 2 to 3 volumes of 0.5 per cent mercuric acetate as in step (3).

8. Treat the washed mercuric nucleotide precipitate with  $\text{H}_2\text{S}$  exactly as in step (4).

9. Then add  $\text{N NaOH}$  slowly, with stirring, until a small amount of precipitate forms. This is  $\text{Ba}_2\text{ATP}$  resulting from a slight amount of barium which is carried through the mercury precipitation and sulfide treatments. If no precipitate forms when the pH has reached 6 or thereabouts, it may be necessary to add a drop or two of 0.5 M barium acetate, but this is very unlikely. In any case only a very small amount of precipitate should be allowed to form. Centrifuge off this precipitate and discard. It will carry down colloidal sulfides.

10. Adjust the pH of the supernatant from step (9) to pH 7.0 by adding more  $\text{N NaOH}$ , and, after diluting the solution to 500 ml., precipitate the barium salt of ATP exactly as in step (5), using the same amount of barium acetate as in step (5). Allow the precipitate to settle (in the ice chest), decant, and centrifuge. Then wash the precipitate twice with ice-water exactly as in step (5). Next wash the precipitate once with 50 per cent ethyl alcohol, once with 75 per cent alcohol, twice with 95 per cent alcohol, and twice with diethyl ether, using adequate volumes of the wash liquid to insure good washing. The precipitate should always be recovered from the wash liquid by centrifugation rather than by filtration. Finally, remove the precipitate from the centrifuge tubes, allow it to dry on hard filter paper, pulverize, and place in a desiccator over  $\text{P}_2\text{O}_5$  for 2 or 3 days before analysis. The dried product will contain 4 molecules of water per molecule of  $\text{Ba}_2\text{ATP}$ . If the  $\text{Ba}_2\text{ATP}$  is dried under high vacuum by means of a Megavac pump for 2 or 3 days, an anhydrous product is obtained.

It has been found possible to obtain approximately an additional 1 gm. of ATP per kilo of muscle if all of the solutions containing the mercury or the barium salt of ATP are allowed to stand in the ice box for an additional length of time. The precipitates are collected and put through whatever part of the procedure that has not yet been carried out. For instance, additional precipitate from the original trichloroacetic acid extract must be dissolved and reprecipitated as barium, then mercury, and then barium salts, while material collected after precipitation the second time as mercury salt need only be precipitated once more as a barium salt. The purity of the additional ATP collected in this way appears to be as high as that of the main fraction.

#### *Preparation of Adenosine Triphosphate from Yeast*

Fresh bakers' yeast is washed twice with distilled water in the centrifuge. A rather low speed must be employed, so that the light cells and debris are

discarded. About 10 per cent of the yeast material is thereby discarded at the first washing. This procedure is necessary to remove colloidal material which would subsequently render impossible the isolation of pure ATP.

The rest of the procedure is the same as the procedure for isolating ATP from muscle, except that 20 per cent trichloroacetic acid is substituted for 8 per cent trichloroacetic acid.

*Preparation of Adenosine Triphosphate Containing Radioactive Phosphorus*

10 million counts of P<sup>32</sup> in less than a mg. of sodium phosphate were injected intravenously into a rabbit. The animal was then caused to exercise strenuously by placing it in a tub of water and allowing it to swim for 30 minutes. After this, the animal was allowed to rest for 2½ hours.

TABLE I

*Specific Radioactivity of Phosphate Groups of Adenosine Triphosphate*

Labile phosphate was hydrolyzed in 1 M HCl for 7 minutes at 100° and separated from the stable phosphate by precipitation with barium acetate. Two of the three phosphates of ATP are hydrolyzed and separated by this procedure.

Material counted	Activity, counts per min. per micromole
ATP, before hydrolysis.....	274
Labile P.....	228*
Stable ".....	48
Total activity recovered after separation.....	276

\* Calculated for the 2 molecules of labile P.

Barium ATP was isolated in the usual manner from the animal by the procedure already described. The animal was prepared by the use of nembutal and curare. The recovery of radioactive phosphate in the ATP, as well as the specific activity of the phosphate groups, is given in Table I. These results are essentially in agreement with those of Flock and Bollman (16), except that our recovery of radioactive phosphate in the ATP was considerably higher.

*Analysis of Adenosine Triphosphate*

The purity of the adenosine triphosphate prepared as described above has been investigated by carrying out a number of analyses on various preparations from rabbit muscle and yeast.

The results of analyses are summarized in Tables II, III, and IV. In addition, analyses which have been carried out by P. S. Krishnan in the laboratory of Professor J. B. Sumner at Cornell University, using barium

TABLE II  
*Analysis of Adenosine Triphosphate*

Constituent	Found	Theoretical for Ba <sub>3</sub> ATP·4H <sub>2</sub> O
	<i>per cent</i>	<i>per cent</i>
Nitrogen.....	8.0	8.20
Barium.....	33.0	32.17
Water.....	8.0	8.47
Hydrolyzable phosphate (as P)*.....	7.3	7.26
Total phosphate (as P)*.....	11.0	10.88
Ratio, total phosphate to hydrolyzable phosphate.....	1.53	1.50

Inorganic phosphate, which was not determined, is included in these figures.

TABLE III  
*Analysis of Second Lot of Adenosine Triphosphate*

Constituent	Found	Theoretical for Ba <sub>3</sub> ATP·4H <sub>2</sub> O
	<i>per cent</i>	<i>per cent</i>
Nitrogen	8.28	8.20
	8.31	
Average	8.30	
Inorganic phosphate (as P)	0.20	0.0
	0.19	
Average	0.20	
Hydrolyzable phosphate (as P)*	7.25	7.26
	7.22	
	7.26	
Average	7.24	
Total phosphate (as P)*	10.80	10.88
	10.98	
	11.08	
Average	10.95	
Ratio, total phosphate to hydrolyzable phosphate	1.51	1.50

\* Inorganic phosphate is not included in these figures.

ATP prepared by the method described in this paper, have yielded similar results. The yields reported by Krishnan were slightly higher than those reported by us.

The analyses for hydrolyzable and total phosphate which are recorded in Tables II and III were carried out following hydrolysis of the sample for 7 minutes in  $N$  HCl or digestion in concentrated sulfuric acid, by a modification of the method reported by Sumner (17). In this modified method, 3.0 ml. of 7.5  $N$   $H_2SO_4$  were used instead of 5.0 ml., and care was taken to have the same concentration of other acid (HCl) in the standard as in the unknown.

TABLE IV  
*Analyses of Other Lots of Adenosine Triphosphate*

Lot No.	Total amount of fresh tissue	Yield* of ATP, gm. per kilo fresh muscle	Hydrolyzable phosphate (as P); theoretical† for $Ba_2$ ATP, 7.86	Total phosphate (as P); theoretical† for $Ba_2$ ATP, 11.8	Nitrogen; theoretical† for $Ba_2$ ATP, 9.0	Barium; theoretical† for $Ba_2$ ATP, 35.3	Pentose; theoretical† for $Ba_2$ ATP, 19.3
	gm.						
1. Rabbit muscle.....	800	1.97	7.80	11.6		35.0	
2. " " .....	950	2.27	7.72	11.7		34.9	
3. " " .....	900	2.07	7.73	11.8	8.75	35.3	19.5
4. " " † .....	2000	2.65	7.60	11.6	8.87	35.3	19.1
5. Yeast.....	458	1.22	7.60	11.7	8.87	35.3	19.2
Average.....			7.69	11.7	8.83	35.1	19.3
% of theoretical....			97.9	99.2	98.1	99.4	100.0

No inorganic phosphate (as P) was found.

\* In all of these preparations with rabbit muscle, 10 per cent trichloroacetic was used instead of 8 per cent. The latter gives somewhat better yields and is optimal in concentration for extracting the ATP.

† The theoretical results are based on  $Ba_2$  ATP. Water was removed before analysis by evacuating in a vacuum desiccator over  $P_2O_5$  with a Megavac pump to a pressure considerably less than 0.1 mm. for 36 hours and allowing to stand under a vacuum for several weeks in the desiccator.

‡ Spectrochemical analyses performed by L. Steadman of the Department of Radiology indicate that the mercury content of this batch of ATP was less than 1 molecule of Hg per 5000 molecules of ATP.

Inorganic phosphate was determined on an aliquot of the stock ATP solution, immediately after it was made up, by the same method.

Nitrogen was determined by the micro-Kjeldahl method with a copper-selenium catalyst.

Barium was determined by precipitating as barium sulfate and weighing.

Water was determined by the loss of weight of a sample after drying over  $P_2O_5$ , with boiling toluene in an Abderhalden apparatus.

The analyses reported in Table IV were carried out as follows: phos-

phates, a modification of the Fiske and Subbarow method ( (18), (19) p. 165); nitrogen, a micro-Kjeldahl method according to Johnson (20); barium, determined gravimetrically according to the method of Kolthoff and Sandell (21); pentose, by a modification of the method of Mejbaum ( (22), (19) p. 166).

The absorption spectrum of ATP prepared according to our directions is shown in Fig. 1.<sup>2</sup> The absorption coefficient of  $1.62 \times 10^4$  is in agreement with that obtained by Kalekar (23).

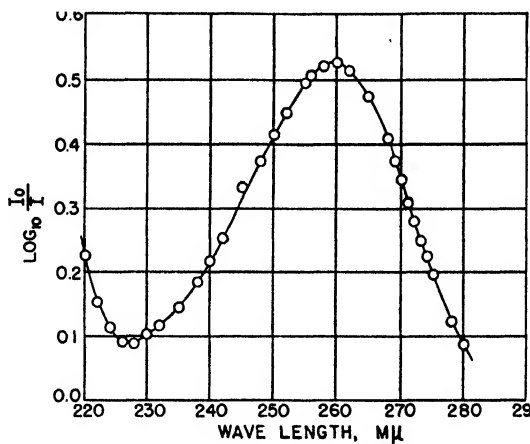


FIG. 1. Absorption spectrum of adenosine triphosphate. Concentration ( $C$ ) =  $3.25 \times 10^{-5}$  M.  $\epsilon_{260 \text{ m}\mu} = (1/C) \times \log_{10} (I_0/I) = 1.62 \times 10^4$ .

#### DISCUSSION

We have described a relatively simple chemical procedure for the isolation of the barium salt of adenosine triphosphate from muscle and yeast which yields a fair amount of product (about 2.5 to 3.0 gm. per kilg of fresh undried muscle) in a highly purified state. There are no new principles involved in the method, but the technical procedure has been refined and standardized and is described in sufficient detail so that it is easily repeated in the hands of graduate students at The University of Rochester and Cornell University.

The amount of radioactive phosphate recovered in ATP prepared from the rabbit injected with radioactive phosphate is remarkably high (about 18 per cent of the dose). It is of interest that the specific activity of the two terminal phosphates in the radioactive ATP was considerably higher than that of the inner phosphate (see Table I).

<sup>2</sup> We are indebted to Dr. A. A. Pihl for determining the absorption spectrum of ATP prepared by our method.

## SUMMARY

1. An improved method has been described for the preparation of highly purified adenosine triphosphate (ATP) from rabbit muscle and yeast.

2. The preparation of ATP containing radioactive phosphate has been described.

3. Analytical data have been furnished for a number of samples of ATP prepared in different laboratories by the method described in this paper.

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# GROWTH INHIBITION OF NEUROSPORA BY CANAVANINE, AND ITS REVERSAL\*

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Canavanine, an amino acid from jack beans, was discovered by Kitagawa and coworkers in 1929 (1, 2). The substance is not combined in the proteins of the seed, but occurs in the free state, and makes up 2.5 per cent of the dry weight of jack beans (3). In a series of papers available to the authors for the most part in abstract only, the Japanese workers have reported extensive investigations into the chemistry and physiology of the substance. The structure of canavanine was established by Gulland and Morris (4) and by Kitagawa and Takani (5) as  $\text{NH}_2 \cdot \text{C}(:\text{NH}) \cdot \text{NH} \cdot \text{O} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CHNH}_2 \cdot \text{COOH}$ . Natural canavanine is of the L configuration (6).

Canavanine is split by a liver enzyme to yield urea and canaline,  $\text{NH}_2 \cdot \text{O} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CHNH}_2 \cdot \text{COOH}$  (3). More recent evidence has indicated that the canavanine-splitting enzyme may be identical with arginase (7). It has been claimed by Ogawa ((8) and elsewhere) that canavanine is essential for young rats. The amino acid is non-toxic to mice, but produces symptoms of intoxication when injected into dogs in a dose of 200 to 400 mg. per kilo of body weight (9).

In experiments designed to test the effectiveness of canavanine in supporting the growth of certain amino acid-requiring mutants of *Neurospora* we found instead that the substance exerts a strong inhibitory effect on the growth of the mold. Further investigation has revealed a number of interesting aspects of this phenomenon, among which are the high degree of toxicity of the substance for the mold, the complete reversibility of the inhibition under certain conditions, and the existence of a genetic factor determining sensitivity or tolerance.

## Materials and Methods

The strains of mold used in this study are 1A, 4A, and 25a. All are wild type strains of *Neurospora crassa*, derived originally from single

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ascospores, and grow normally on the usual minimal medium containing sugar, salts, and biotin (10). Growth was measured as the dry weight of mold produced in 72 hours at 25° in 20 ml. of medium contained in 125 ml. Erlenmeyer flasks.

Canavanine flavianate was isolated from jack bean meal by the method of Gulland and Morris (4). The flavianate was decomposed according to the procedure of Cadden (6), and canavanine was obtained as the sulfate. Electrometric titration and elementary analysis of the salt indicate the constitutional formula  $(C_6H_{12}N_4O_3)_2 \cdot H_2SO_4$ .

Calculated.	C 26.65,	H 5.75,	N 24.90,	sulfate S 7.11;	equivalent wt. 225
Found.	" 27.21,	" 6.31,	" 24.50,	" " 7.05;	" " 229

### Results

In contrast to other natural amino acids which have been shown to produce inhibition of growth in *Neurospora* (11–13), canavanine is effective against the wild type, as well as against mutant strains. Different degrees of sensitivity are exhibited by different wild strains, however. Following the initial observation that canavanine retards the growth of strain 1A, a number of other wild types were tested. It was found that three grades of resistance to the action of the amino acid can be distinguished: a high degree of resistance, shown by strain 4A; medium resistance, shown by strain 1A; and low resistance, shown by strain 25a. In Fig. 1 are plotted typical experiments showing the growth of these strains as a function of canavanine concentration. It is seen that growth of strain 25a is abolished by concentrations of canavanine sulfate exceeding 1.25  $\gamma$  per ml. ( $5.55 \times 10^{-6}$  M with respect to canavanine). With strain 1A a 10-fold greater concentration is required to bring about a 55 per cent inhibition of growth, while strain 4A is inhibited to the extent of only 15 per cent by the highest concentration tested.

Although the responses of strains 25a and 1A to canavanine are quite reproducible, strain 4A has not shown the same degree of tolerance to the substance in all experiments. In some tests it has behaved very much as strain 1A (medium resistance), while in others it has shown absolute resistance. The results of a preliminary experiment have indicated that the age of the culture is probably a factor in determining the response of strain 4A, resistance increasing with age.

In the present experiments the standard incubation period of 72 hours was used. If the culture flasks are allowed to incubate for longer periods, growth of strain 25a will eventually begin, even in the presence of relatively high canavanine concentrations. Whether this is caused by a loss of canavanine through spontaneous decomposition to desaminocanavanine (14), or by a change in the mold, we are not yet prepared to say.

**Reversal of Canavanine Inhibition**—On the assumption that canavanine interferes with the production or utilization of an essential metabolite, the growth of strain 1A was measured in the presence of an inhibiting concentration of canavanine (50  $\gamma$  of canavanine sulfate per ml.) plus various supplements. In a preliminary experiment it was found that a mixture of water-soluble vitamins has only a slight effect on the inhibition, while hydrolyzed casein in a concentration of 1.25 mg. per ml. of medium completely reverses it. Two mixtures of amino acids were then tested, one containing Rose's essential amino acids, the other the "non-essential" amino acids. Canavanine inhibition was abolished by the mixture of

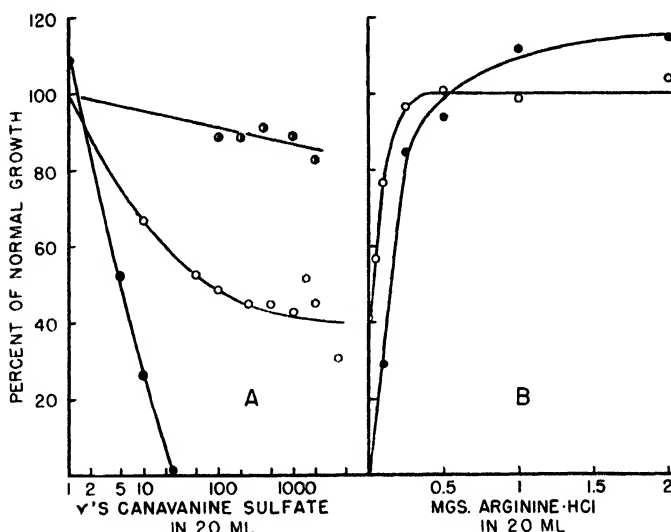


FIG. 1. A, inhibition of the growth of *Neurospora* by canavanine. ●, strain 4A; ○, strain 1A; ●, strain 25a. B, reversal of inhibition by arginine. Canavanine sulfate concentration, 0.25 mg. per 20 ml. in the strain 25a curve (●) and 1 mg. per 20 ml. in the strain 1A curve (○).

essential amino acids (final concentration, 0.05 mg. of each per ml.), while the non-essential mixture produced a small effect (final concentration, 0.1 mg. of each per ml.).

The ten amino acids making up the essential mixture were then tested singly, with the results indicated in Table I. It is seen that complete reversal of the inhibition was obtained only with arginine; lysine and methionine were moderately effective (60 per cent reversal), while the remaining amino acids showed small activities.

When the same series of amino acids was tested on strain 25a, it was found again that the inhibition is completely relieved by arginine. Lysine

is also effective in this strain, with an activity equal to 0.25 that of arginine on a molecular basis. None of the other amino acids showed any activity (Table I). Typical recovery curves are plotted in Fig. 1. It is calculated that the effect of 1 molecule of canavanine is neutralized by about 3 molecules of arginine in strain 25a and by 0.3 molecules of arginine in strain 1A.

Experiments were next carried out to determine the relative effectiveness of optical isomers of arginine and lysine in overcoming canavanine inhibition. The results show that L-arginine and L-lysine are twice as active as the racemic mixtures in protecting strain 25a from the inhibition (Table II). It is concluded that only the natural enantiomorphs are active in

TABLE I  
*Effect of Essential Amino Acids on Canavanine Inhibition*

Concentration of canavanine, M/4500 (50  $\gamma$  of canavanine sulfate per ml.) in the strain 1A series and M/10,500 (21.4  $\gamma$  of canavanine sulfate per ml.) in the strain 25a series. Concentration of other amino acids, 0.1 mg. per ml. in the strain 1A series and M/400 in the strain 25a series.

Supplement	Strain 1A, growth	Strain 25a, growth
	mg.	mg.
None .....	72	53
Canavanine .....	14	0
“ + L-arginine·HCl .....	80	54
“ + DL-lysine·HCl .....	49	46
“ + DL-leucine .....	27	0
“ + DL-isoleucine .....	33	0
“ + L-methionine .....	47	0
“ + DL-valine .....	36	0
“ + DL-phenylalanine .....	27	0
“ + L-tryptophan .....	27	0
“ + DL-threonine .....	27	0
“ + L-histidine·HCl·H <sub>2</sub> O .....	25	0

this respect. This is somewhat surprising in view of the fact that *arginineless* mutants of *Neurospora* utilize DL-arginine for growth just as readily as L-arginine (15).

In another series of experiments, the effect on the growth of strain 25a of simultaneously varying the canavanine and arginine concentrations was measured. The results are presented in Table III. It will be noted that the degree of inhibition is independent of the absolute concentrations of the two amino acids, but is determined solely by the ratio of the concentrations. Inhibition quotients (ratio of canavanine concentration to arginine concentration) calculated from these data are close to 1.2 for complete inhibition and 0.3 for complete reversal.

Several miscellaneous guanidine derivatives have been tested for relief of canavanine inhibition, including guanidine, creatine, carbamidoarginine, guanidine valeric acid, guanidine butyric acid, and benzoylargininamide. The last two compounds were kindly supplied by Professor Carl Niemann. None of these substances was found to be active by itself. If the

TABLE II

*Effect of Optical Isomers of Arginine and Lysine on Canavanine Inhibition*

The values represent growth of strain 25a in mg. Canavanine concentration, M/10,500 throughout.

Arginine or lysine concentration	L-Arginine	DL-Arginine	L-Lysine	DL-Lysine
M/800	70.0	64.0	62.5	47.5
M/1600	64.0	60.5	48.0	18.5
M/3200	58.5	42.0	21.0	0.0
M/6400	42.0	5.0	0.5	0.0
M/12,800	4.5	0.0	0.0	0.0
M/25,600	0.0	0.0	0.0	0.0

TABLE III

*Growth of Strain 25a on Independently Varying Concentrations of Arginine and Canavanine*

The values represent growth in mg.

Arginine concentration	Canavanine concentration						
	0	M/42,000	M/21,000	M/10,500	M/5250	M/2625	M/1313
M/200	54.0	49.5	52.0	50.0	50.0	47.0	59.0
M/400		49.0	58.0	52.5	58.5	51.0	45.5
M/800		49.5	48.5	47.0	46.5	45.0	17.5
M/1600		50.5	49.5	54.5	50.0	24.0	2.0
M/3200		49.5	54.5	54.0	31.5	4.0	0.0
M/6400		56.0	53.0	37.5	9.5	0.0	0.0
M/12,800		50.5	34.0	4.0	0.0	0.0	0.0
M/25,600		33.0	5.0	0.0	0.0	0.0	0.0
M/51,200		4.0	0.0	0.0	0.0	0.0	0.0
0	50.0	0.0	0.0	0.0	0.0	0.0	0.0

medium is supplemented with just enough arginine to provide for a small amount of growth, then the further addition of carbamidoarginine produces a marked increment in the growth and guanidine a slight increase. The activity of carbamidoarginine is probably ascribable to its conversion to arginine by the mycelium, since experiments with *arginineless* mutants have shown that it supports the growth of these strains.

Experiments have been carried out to determine whether changes in temperature and pH affect the inhibition. Flasks containing an inhibitory concentration of canavanine ( $9.5 \times 10^{-5}$  M) in basal medium were inoculated with strain 25a and incubated at 20°, 25°, 30°, and 35°, respectively. No growth was observed in any of the flasks after 78 hours. In another series of flasks, medium buffered at six pH values in the range 4.8 to 6.9 and containing canavanine in a concentration of  $4.75 \times 10^{-5}$  M was inoculated with strain 25a and incubated at 25° for 72 hours. Again, no relief of the inhibition was observed.

*Genetics of Canavanine Tolerance*—The differences between various wild type strains with respect to canavanine tolerance made it of interest to investigate the inheritance of this character. Strain 25a was crossed with strain 4A and the spores from eighteen asci were isolated in order and transferred to agar slants. The resulting cultures were then tested for canavanine tolerance. In thirteen of the asci canavanine tolerance segregated in a manner indicating that tolerance and sensitivity are determined by alternative forms of a single gene. The remaining five asci, however, could not be so simply interpreted and it is evident that a more extensive series of crosses will be necessary in order to establish the mode of inheritance of this pair of characters.

Dr. H. J. Teas has analyzed a number of other crosses of resistant and sensitive strains, with results essentially similar to ours.

#### DISCUSSION

Perhaps the most remarkable feature of the inhibition by canavanine is the extraordinarily high degree of toxicity displayed by this compound, an amino acid of the natural series. Not only is canavanine effective in very low concentrations (order of  $10^{-6}$  M), but the neutralization quotients 0.3 and 1.2 obtained above contrast markedly with the values 100 to 10,000 usually found for metabolic antagonists (16). It is of interest to note that the ability to synthesize arginine and lysine, the only effective antidotes so far discovered, is not protective; the strains used in this study are wild types capable of synthesizing all of the amino acids necessary for their normal growth from sugar and inorganic salts. This suggests that the relative immunity to canavanine intoxication displayed by strain 4A results from a mechanism for detoxifying or otherwise disposing of the compound, a mechanism presumably possessed by strain 25a in a much less active form, if at all. This would be analogous to the case described by Woolley (17), who found that certain pyrithiamine-fast microorganisms possess a system for destroying pyrithiamine, whereas sensitive strains do not.

Little can be said at present as to the mechanism of the inhibition.

Although the data suggest a competition between arginine and canavanine for an enzyme surface, it is difficult on this basis to explain the anticavanine action of lysine. The present observations would appear to be related in some way to the lysine-arginine antagonism discovered by Doermann (11). One possible mechanism seems to be definitely excluded by our data; namely, the interference of canavanine with arginine synthesis. On this basis, one would expect to provide complete protection against canavanine by supplying sufficient arginine in the medium for normal growth requirements. Studies of *arginineless* mutants (15) have shown that arginine in a concentration of  $m/2000$  is sufficient for good growth. As can be seen in Table III, however,  $m/1600$  arginine is not protective against the inhibition.

We wish to thank Dr. G. Oppenheimer and Dr. A. J. Haagen-Smit for the microanalyses of C, H, and N.

#### SUMMARY

L-Canavanine from jack beans is a powerful inhibitor of the growth of certain wild type strains of *Neurospora*. Growth of the most sensitive strain is abolished by canavanine in a concentration of  $5.55 \times 10^{-8}$  M. Growth of a second strain is partly inhibited by canavanine, while a third strain is almost completely resistant. Resistance and sensitivity appear to be genetically determined. The inhibition is reversed by L-arginine and, in one of the strains, by L-lysine. The antagonism between canavanine and arginine is of the "competitive" type, approximately 3 molecules of arginine being required to neutralize the effect of 1 molecule of canavanine in the most sensitive strain. Lysine is about 0.25 as active as arginine for this strain. Although the mechanism of the inhibition is not known, the data exclude the possibility of interference by canavanine with arginine synthesis.

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## LETTERS TO THE EDITORS

### RELEASE OF ANTIBODY BY LYMPHOID TISSUE IN VITRO\*

*Sirs:*

Studies have been made of the release of antibodies during incubation of tissues from rats and mice immunized to sheep erythrocytes. Tissue minces were shaken at 38.5° in Warburg type flasks through which was passed a constant stream of 95 per cent oxygen-5 per cent carbon dioxide. The incubation medium was serum obtained from non-immunized animals of the same strain, age, and sex. After 3 hours incubation, comparison was made of the antibody titer and the amount of protein released by the tissue in each flask. A separate aliquot of the same tissue was ground with sand

Tissue mince	Antibody release value (1)	Antibody extraction value (2)	Incubation selectivity ratio, (1)/(2)
Liver.	13.2 (16)*	20.1 (64)	0.7
Spleen.	13.8 (16)	28.7 (64)	0.5
Kidney.	8.8 (8)	15.0 (32)	0.6
Lymphoid tissue.	166.3 (16)	3.1 (4)	53.2

\* The values in parentheses are reciprocals of hemolysin titers (doubling dilution method).

in physiological saline, and the antibody titer and protein nitrogen of the extract determined. In the table, antibody release is expressed as the ratio of the reciprocal of the titer to the number of mg. of protein nitrogen added to each ml. of serum during incubation. Similarly, antibody extraction is expressed as the reciprocal of the titer in the saline extract of a tissue, divided by the number of mg. of protein nitrogen in each ml. of the extract. The incubation selectivity ratio is equal to the antibody release value divided by the antibody extraction value. The magnitude of the incubation selectivity ratio indicates the extent of selective release of antibody during incubation.

Minces of lymphoid tissue (pooled thymuses and mesenteric nodes) of immunized mice released agglutinins and hemolysins against sheep erythrocytes in higher titers, relative to the amount of protein added to the serum

\* This investigation was aided by grants from the American Cancer Society on recommendation by the Committee on Growth of the National Research Council, and from the Josiah Macy, Jr., Foundation.



during incubation, than either liver, spleen, or kidney minces. Agglutinin determinations were early abandoned, since agglutinating activity against sheep erythrocytes was found in saline extracts of non-immune mouse and rat tissues; this difficulty was not encountered with hemolysin determinations.

The table presents the results of a typical experiment. Tissues were obtained from adult, male Sprague-Dawley rats exsanguinated under nembutal anesthesia. These animals were previously immunized by intraperitoneal injections of 1 ml. of a 10 per cent suspension of sheep erythrocytes (ten injections during 2 weeks). The incubation selectivity ratio is significantly large only for lymphoid tissue.

Similar experiments have also been performed with tissues obtained from rats 5 days after a single intravenous injection of 0.5 ml. of a 2 per cent suspension of sheep erythrocytes. The results suggest that the behavior of lymphoid tissue and spleen in releasing antibody *in vitro* differed significantly with the method of immunization employed. Studies of thymus and mesenteric lymph nodes separately indicated that the capacity for selective release of antibody *in vitro* by lymphoid tissue resides chiefly in the lymph nodes. When a nitrogen atmosphere replaced the oxygen-carbon dioxide mixture, the antibody release value was the same as the antibody extraction value.

Splenic tissue from rats given a single intravenous injection of antigen had relatively high hemolysin titers both before and after incubation as did the serum medium in which the tissue was incubated. In several experiments, the titer of antibody released by spleen during incubation, plus that extractable from the tissue after the incubation period, was significantly greater than that demonstrable in splenic extracts prior to incubation. The results suggest that antibody production may have occurred *in vitro*.

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## DIMETHYL- $\beta$ -PROPIOTHETIN, A NEW METHYL DONOR

*Sirs:*

Studies of the ability of various compounds to promote the growth of rats on methyl-free diets supplemented with homocystine have established that a marked structural specificity is involved.<sup>1</sup> In this earlier report the only N-methyl compounds found to be capable of acting as direct methyl donors were choline, dimethylethyl- $\beta$ -hydroxyethylammonium chloride, and betaine. During the course of testing other compounds structurally related to choline and betaine for growth-promoting activity under these conditions, it was found in this laboratory that dimethylthetin,  $(\text{CH}_3)_2\text{SCH}_2\text{COO}^-$ , the sulfur analogue of betaine, was also very effective and able

Diet	Rat No.	Diet period	Weight change over period	Food consumption	Growth rate
		<i>days</i>	<i>gm.</i>	<i>gm. per day</i>	<i>gm. per day</i>
Basal diet	25	21	113- 95	5.0	-0.9
	28	21	97- 89	4.8	-0.4
	50	12*	81- 71	3.4	-0.8
" " + 0.5% choline	24	21	108-165	8.8	+2.7
	49	23	95-141	8.3	+2.0
	51	21	85-153	9.2	+3.2
" " + 0.92% di- methylpropiothetin	41	21	98-158	8.8	+2.9
	48	21	88-137	7.9	+2.3
	57	21	101-180	9.3	+3.8

\* Rat died.

to support growth about as well as choline itself.<sup>2, 3</sup> The rôle of dimethylthetin as a methyl donor has been further explored *in vitro* by Dubnoff and Borsook.<sup>4</sup>

Recently Challenger and Simpson<sup>5</sup> reported the isolation of dimethylpropiothetin,  $(\text{CH}_3)_2\text{SCH}_2\text{CH}_2\text{COO}^-$ , from a marine alga, *Polysiphonia fastigiata*. Since this compound is a homologue of dimethylthetin, it occurred to us that it might also be an active methyl donor, and this has proved to be the case. In the table are shown the average growth rates of rats on a basal diet<sup>6</sup> containing no methionine and 0.87 per cent of homocystine, and supplemented with 0.92 per cent of dimethylpropiothetin

<sup>1</sup> Moyer, A. W., and du Vigneaud, V., *J. Biol. Chem.*, **143**, 373 (1942).

<sup>2</sup> du Vigneaud, V., *Harvey Lectures*, **38**, 39 (1942-43).

<sup>3</sup> du Vigneaud, V., Moyer, A. W., and Chandler, J. P., *J. Biol. Chem.*, in press.

<sup>4</sup> Dubnoff, J. W., and Borsook, H., *Federation Proc.*, **7**, 152 (1948).

<sup>5</sup> Challenger, F., and Simpson, M. I., *Biochem. J.*, **41**, p. xl (1947).

chloride. For comparison are given corresponding data for the animals on a similar diet containing 0.5 per cent choline instead of the thetin, and for those on the basal diet containing no methyl donor.

The animals on the diet containing dimethylpropiothetin grew as well as those on the diet containing choline and remained in excellent condition throughout the experiment. There was no kidney enlargement detectable at any time, and on autopsy there were no signs of hemorrhagic damage to the kidneys as there were in the case of the animals on the basal diet. Estimation of total liver lipides gave values which agreed closely with those from the rats fed choline.

The significant discovery of this compound as a naturally occurring substance, coupled with our findings of the pronounced growth-promoting activity of both dimethylthetin and dimethylpropiothetin, makes it appear possible that these substances, or closely related derivatives, may be present in animal tissues and may take part in normal methylation processes. A more detailed study, including data for other related compounds, is to be published in due course.

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# SYNTHESIS OF NICOTINIC ACID FROM TRYPTOPHAN BY THE DEVELOPING CHICK EMBRYO\*

*Sirs:*

Recent investigations with several species of animals have revealed that tryptophan can be converted into nicotinic acid within the tissues of the animals, by the intestinal microorganisms, or by a combination of these mechanisms. In the present investigation more direct evidence has been obtained to demonstrate that this conversion can occur within the tissues in the absence of the complicating effects of the intestinal flora. Developing chick embryos were selected for these tests. Eggs from hens maintained on a standard diet were incubated for a period of 7 days and injected with 2 ml. of a sterilized solution containing 30 mg. of L-tryptophan dis-

Treatment	Nicotinic acid content*	
	$\gamma$ per gm.	Total mg.
Controls . . . . .	14.1 (13)	0.91
Tryptophan . . . . .	17.5 (16)	1.13

Statistical analysis		
F value observed . . . . .	17.98	20.25
" " required (1% level) . . . . .	7.68	7.68

\* The number of embryos analyzed is given in parentheses.

solved in 0.9 per cent saline adjusted to neutrality. Eggs injected with 2 ml. of 0.9 per cent saline served as controls. The injection techniques devised were such as to minimize injury to the embryo and to minimize bacterial infections which may result from the manipulations used. After injection, the eggs were incubated for an additional 10 days. The entire contents of each egg were then homogenized in a Waring blender with an amount of water equal to the original weight of the egg before incubation. Aliquots of the homogenates were autoclaved with an equal amount of 2 N H<sub>2</sub>SO<sub>4</sub> for 30 minutes and, after appropriate preparation of the samples, the nicotinic acid content was determined microbiologically. Only those embryos that were alive at 17 days were taken for analysis. A summary of the results obtained for two separate experiments is presented in the accompanying table.

\* Acknowledgments are made to Frances Panzer for valuable technical assistance. This work was supported in part by a grant from Swift and Company.

A statistical analysis of the data showed that the addition of tryptophan increased the nicotinic acid content and that the results are highly significant ( $P < 0.01$ ). The values obtained for the controls are in excellent agreement with those reported by Snell and Quarles<sup>1</sup> for chick embryos of the same age.

It may be concluded, therefore, that nicotinic acid is synthesized from tryptophan within the tissues as evidenced by the results obtained with the developing chick embryo. The results obtained substantiate the suggestions that tryptophan serves as a precursor of nicotinic acid synthesis in the chick,<sup>2</sup> turkey,<sup>3</sup> and other animals.<sup>4</sup>

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<sup>1</sup> Snell, E. E., and Quarles, E., *J. Nutr.*, **22**, 483 (1941).

<sup>2</sup> Briggs, G. M., Groschke, A. C., and Lillie, R. J., *J. Nutr.*, **32**, 659 (1946).

<sup>3</sup> Furman, C., Snell, E. E., and Cravens, W. W., *Poultry Sc.*, **26**, 307 (1947).

<sup>4</sup> Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, **166**, 573 (1946). Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, **168**, 555 (1947); **172**, 485 (1948). Henderson, L. M., Deodhar, T., Krehl, W. A., and Elvehjem, C. A., *J. Biol. Chem.*, **170**, 261 (1947).

## BIREFRINGENCE OF FLOW OF PREPARATIONS OF MYOSIN\*

*Sirs:*

Mommaerts<sup>1</sup> has reported that myosin, prepared according to Szent-Györgyi, has an extinction value of  $12^\circ$ , and that actomyosin orients parallel to the optical axis when studied in the birefringence of flow apparatus. Von Muralt and Edsall<sup>2</sup> many years earlier had reported an extinction value of  $12^\circ$  for myosin preparations now presumed to have been actomyosin. During the course of an extensive study of the physical properties of the myosin complex we have had occasion to study birefringence of flow in an apparatus similar to the one described by Edsall *et al.*<sup>3</sup> The results we have obtained appear to reconcile the previous observations.

Crystalline myosin, obtained by an adaptation of the methods of Szent-Györgyi<sup>4</sup> and found to be homogeneous in the ultracentrifuge, was found to have an extinction angle of  $22^\circ$  when examined at velocities as great as 1600 R.P.M. Fibrous actin, prepared from crude actomyosin or from acetone-desiccated muscle, was found to orient parallel to the optical axis at velocities as low as 100 R.P.M. When crystalline myosin and purified fibrous actin were mixed in the ratio of 3 parts to 1, on a basis of nitrogen content, the extinction was observed to change over a 2 hour period and at 300 R.P.M. from an initial value of  $35^\circ$  to  $12^\circ$ . If the myosin-actin ratio was increased to 1 or greater, complete orientation was observed. When minute amounts of actin were mixed with crystalline myosin, an extinction of  $12^\circ$  was observed at high velocities. Actomyosin extracted from muscle by the methods of Weber and Meyer<sup>5</sup> was observed to have an extinction of  $12^\circ$  at 300 R.P.M. It thus appeared that the increment in birefringence of flow of actomyosin over that of myosin was related to the actin content; the normal ratio in the actomyosin complex appeared to be approximately 3 parts of myosin to 1 part of actin.

The birefringence of flow of crystalline myosin and actomyosin but not of actin was found to be reduced by the addition of adenosine triphosphate (0.01 per cent solution). The reaction with myosin was monophasic, of small magnitude, and transient; with actomyosin the reaction was biphasic and sustained. The first phase of the reaction of adenosine triphosphate

\* These studies were supported by a grant from the United States Public Health Service.

<sup>1</sup> Mommaerts, W. F. H. M., *Nature*, **156**, 631 (1945).

<sup>2</sup> von Muralt, A. L., and Edsall, J. T., *J. Biol. Chem.*, **89**, 351 (1930).

<sup>3</sup> Edsall, J. T., Gordon, C. G., Mehl, J. W., Scheinberg, H., and Mann, D. W., *Rev. Scient. Instruments*, **15**, 243 (1944).

<sup>4</sup> Szent-Györgyi, A., *Chemistry of muscular contraction*, New York (1947).

<sup>5</sup> Weber, H. H., and Meyer, K., *Biochem. Z.*, **266**, 137 (1933).

and actomyosin appeared to be identical with that observed with myosin. The second phase was sustained and of much greater magnitude; recovery from this phase resembled the combination of actin with myosin. Results of studies of viscosity and ultracentrifugal behavior under similar conditions will be presented in the near future.

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## A NEW MAGNESIUM-ACTIVATED ADENOSINTRIPHOSPHATASE FROM MUSCLE\*

*Sirs:*

Besides the ATPase<sup>1</sup> which is bound to the myosin fraction,<sup>2</sup> the muscle contains a second powerful ATPase which can be completely separated from myosin. Although it is mainly responsible for the ATPase activity of fresh muscle extract,<sup>3</sup> it was overlooked owing to its instability. It is best ob-

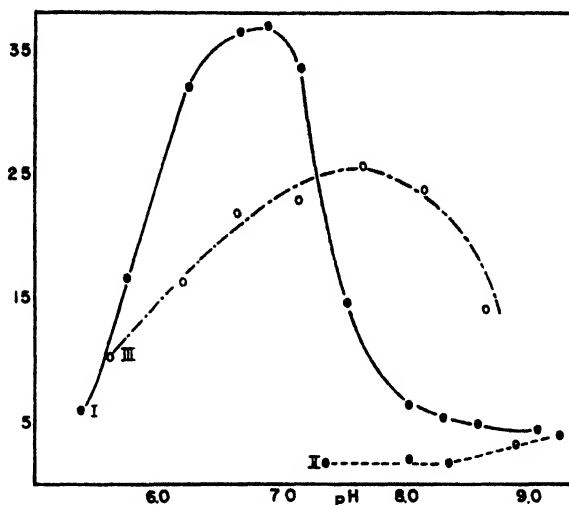


FIG. 1. Ordinate,  $Q_p$  in hundreds. Mg concentration = 0.005 M. Curve I, a Mg-activated ATPase of rather low activity, in histidine buffer; Curve II, a crystalline myosin preparation, in borate; Curve III, myosin-actin (ratio 4:3) in acetate-veronal.  $Q_p$  calculated on the basis of myosin only.

tained by extraction with fairly alkaline KCl solution (0.5 M, pH 10.5, carbonate buffer), dilution with 6 volumes of water, and precipitation of the supernatant (after centrifugation) with 0.35 saturated ammonium sulfate. Precipitation is repeated and the enzyme is further purified on precipitation by dilution with  $H_2O$ . Finally the enzyme is dissolved in 0.5 M KCl and centrifuged at  $18,000 \times g$  for  $\frac{1}{2}$  to 1 hour. The active

\* This work was aided by grants from the American Cancer Society and the United States Public Health Service.

<sup>1</sup> ATP, adenosine triphosphate; ATPase, adenosinetriphosphatase, splitting only one labile P group.

<sup>2</sup> Engelhardt, W. A., and Lyubimowa, M. N., *Nature*, **144**, 668 (1939).

<sup>3</sup> Lohmann, K., *Biochem. Z.*, **281**, 264 (1934).



precipitate contains some lipide besides protein. Nucleic acid can be removed without change of activity. Myosin and actomyosin are completely removed in the first steps of this procedure. Centrifugation increases the activity about 3 times.  $Q_p$  (c.mm. of  $H_2PO_4$  per mg. of protein per hour) at pH 7.5 in the presence of 0.005 M Mg lies usually between 7000 and 10,000, but decreases rapidly. The pH-activity curve is shown in Fig. 1. At pH 9.5 the activity is nearly zero. Ca inhibits strongly. The activity is therefore antagonistic to that of myosin. Addition of actin shifts the pH optimum of myosin-ATPase to more neutral values and also elicits some activation by Mg, as had been found by Szent-Györgyi.<sup>4</sup> This is shown in the dotted curves in Fig. 1. However, our Mg ATPase is free of actin and actin has no influence on its activity. 1 gm. of fresh muscle contains about as much of the new enzyme as of myosin ATPase (measured at their pH optima with Mg and Ca respectively). By its indifference to the presence of creatine, it is distinguished from muscle enzyme preparations described by other authors (Engelhardt, Cori).

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<sup>4</sup> Chemistry of muscular contraction, New York, 50 (1947).

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# THE INTERMEDIATE RÔLE OF CARBAMYL-L-GLUTAMIC ACID IN CITRULLINE SYNTHESIS\*

Sirs:

The carbon dioxide fixation reaction in the initial step of the urea cycle has been assumed to involve the direct carboxylation of the  $\alpha$ -amino group

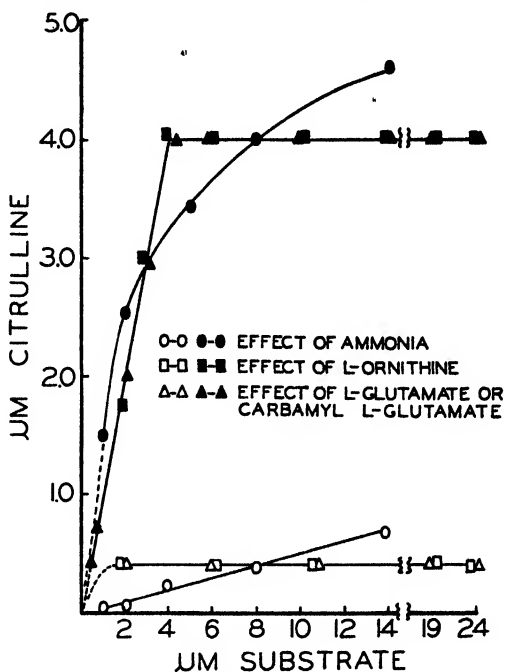


FIG. 1. Final substrate concentrations. Magnesium sulfate  $6 \times 10^{-3}$  M; ATP  $1.2 \times 10^{-3}$  M; AMP  $4 \times 10^{-4}$  M; potassium phosphate buffer at pH 7.15,  $1.3 \times 10^{-3}$  M; fumarate  $2 \times 10^{-3}$  M and potassium ions to bring the medium to isotonicity. Tissue concentration 4.76 mg. of N per flask. In experiments in which any given substrate concentration was not varied, the final concentration of each was as follows: ammonia  $2 \times 10^{-3}$  M; ornithine (calculated on the basis of L-)  $2.5 \times 10^{-3}$  M; carbamyl-L-glutamate  $3.8 \times 10^{-3}$  M; L-glutamate  $3.8 \times 10^{-3}$  M. Incubation time 35 minutes at  $38^\circ$  in air. KOH in center wells. The open symbols represent experiments in which L-glutamic acid is used; the solid symbols, those in which carbamyl-L-glutamic acid is used.

of ornithine.<sup>1</sup> A more detailed study of this reaction, with use of the washed residue from potassium chloride-homogenized rat liver<sup>2</sup> revealed

\* Aided in part by a grant from the Wisconsin Alumni Research Foundation.

<sup>1</sup> Krebs, H. A., in Annual review of biochemistry, Stanford University, 5, 262 (1936).

<sup>2</sup> Cohen, P. P., and Hayano, M., *J. Biol. Chem.*, 172, 405 (1948).

that glutamic acid was acting as the initial acceptor for carbon dioxide.<sup>3</sup> In the study of a large series of compounds which might be expected to behave as intermediates in this reaction, it was observed that carbamyl-L-glutamic acid was highly active in the conversion of ornithine to citrulline. Thus this compound could be shown to be 2 to 3 times more active than L-glutamic acid in citrulline synthesis in the presence of carbon dioxide, and 10 to 15 times more active in the absence of carbon dioxide. Of interest is the finding that free ammonia is necessary for the reaction. The effects of varying concentrations of L-ornithine, ammonia, carbamyl-L-glutamic acid, and L-glutamic acid on citrulline synthesis in a carbon dioxide-free medium are shown in Fig. 1.

In the presence of ammonia, incubation of carbamyl-L-glutamic acid with the enzyme system aerobically followed by the addition of ornithine and anaerobic incubation results in citrulline synthesis. All efforts to date to make the over-all reaction ornithine  $\rightarrow$  citrulline proceed anaerobically have been unsuccessful. While carbamyl-L-glutamic acid behaves in many ways like a true intermediate in the synthesis of citrulline from ornithine, the requirements for free ammonia and aerobic conditions indicate that a closely related carbamino derivative of glutamic acid is involved. It is not excluded that the reaction involves two enzymatic steps, the first of which is the formation of carbamino-L-glutamic acid and the second of which is concerned with the fixation of ammonia.

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<sup>3</sup> Cohen, P. P., and Grisolia, S., *Federation Proc.*, **7**, 150 (1948).

# ANTIPODAL SPECIFICITY IN THE INHIBITION OF GROWTH OF *ESCHERICHIA COLI* BY AMINO ACIDS\*

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(Received for publication, November 28, 1947)

The inhibitory action of D-leucine, D-valine, and DL-valine upon the growth of *Lactobacillus arabinosus* has been demonstrated experimentally (1, 2). Such studies are of interest in cellular chemistry. The specificities observed are related theoretically to those found in the substrates of proteases (3, 4). The information obtained is also pertinent to experiments on the retardation of growth of laboratory animals by nutritional amino acid mixtures (5) and to knowledge of the factors influencing microbiological assay (6). Data of this type may furthermore help to form a picture of the essential structures and modes of action of such antibiotics as gramicidin, tyrocidine, and penicillin (1, 2, 7).

The study of bacterial inhibition by D-amino acids has now been extended to other species in order to determine whether or not the effect may be a special or general one. The effects on *Escherichia coli* reported here for D-valine and D-leucine are similar to those found with *Lactobacillus arabinosus*. A pattern of inhibition of the same sort has been observed for *Staphylococcus aureus* also. In the case of *Staphylococcus aureus*, however, the inhibitions were not consistently observed, although the majority of tests showed inhibition. The nature of this variation is receiving further investigation.

The influences of amino acids on *Escherichia coli* have corresponded closely to the specificity of amino acid residues in substrates of proteases (3). These results differ qualitatively from those observed on *Lactobacillus arabinosus* in the case of D-alanine only. Attempts to clarify the knowledge of the particular function of D-alanine in these organisms were therefore made; data on the competitive behavior of D-alanine are presented below. The effect of D-alanine which has been studied concerns its ability to counteract the inhibition of bacterial growth by glycine. This relationship had previously been observed in *Streptococcus lactis* by Snell and Guirard (8).

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Some phases of this paper were presented before the American Society of Biological Chemists, May, 1947, at Chicago.

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### Materials and Methods

**Amino Acids**—The preparation of the six optical forms of alanine, valine, and leucine has been described (1, 2). The purification of benzoylalanine employed in the resolution of alanine was facilitated by washing out the contaminating benzoic acid with hot carbon tetrachloride, as for hippuric acid (9), instead of with ligroin (10). The glycine used was Merck's amino-acetic acid.

**Growth of Microorganisms**—The growth of *Lactobacillus arabinosus* 17-5 was estimated by titration of acid produced (1).

The growth of *Escherichia coli* was measured turbidimetrically. Stock cultures of *Escherichia coli* were maintained in the refrigerator by monthly transfer to nutrient agar slants (11). The identity of the organism was checked after completion of the experiments (12). Transfers for inhibition tests were made to tubes of nutrient broth (13) and incubated for 24 hours at 37°, centrifuged, washed with physiological saline solution, and resuspended in 10 ml. of saline solution.

The amino acids were included in tubes in 0.50 ml. of aqueous solution, and 0.50 ml. of nutrient broth was added to each tube. These tubes were sterilized for 15 minutes at 15 pounds steam pressure, allowed to cool, and inoculated with 1 drop of the shaken suspension. They were then incubated for 24 hours at 37°, treated with 9.0 ml. of water each, shaken thoroughly, and read in a Coleman model 11 spectrophotometer with Filter PC-4 at 450 m $\mu$ .

Standard dilution curves were determined for each experiment in order to evaluate variations in the rate of growth of the control. These standard curves were prepared by dilution of the incubated control tubes with appropriate amounts of nutrient broth solution which had been diluted to one-twentieth of its original concentration. The spectrophotometer scale was set at 100 per cent transmission with this latter solution.

For the experimental tubes, the galvanometer readings were converted to a calculated value, *growth ratio*, in order to furnish a basis of comparison between sets of experiments. The growth ratio was determined by reading from the corresponding standard dilution curve the per cent of maximum growth (in absence of inhibitors) to which the observed turbidity corresponded.

### EXPERIMENTAL

In Fig. 1 is presented a typical standard curve for the relationship of galvanometer readings and successive dilutions of a control culture of *Escherichia coli*.

In Fig. 2 is presented a typical series of curves illustrating the growth ratios of *Escherichia coli* in the presence of glycine, D-alanine, D-valine, and

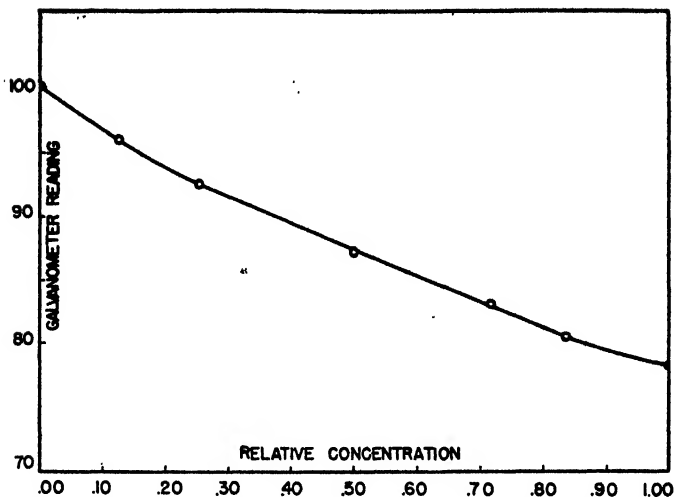


FIG. 1. Graph of galvanometer readings and successive dilutions of a control culture of *Escherichia coli*.

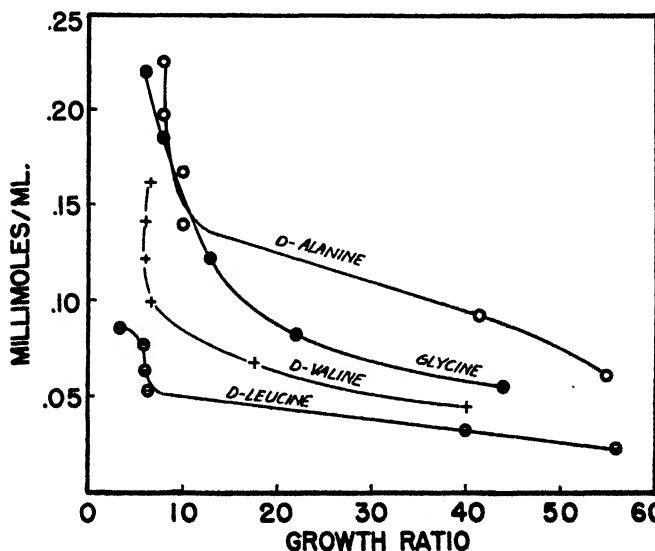


FIG. 2. Effects of varying amounts of D-leucine, D-valine, D-alanine, and glycine on the growth of *Escherichia coli*. The growth ratio was determined by reading from the corresponding standard dilution curve the per cent of maximum growth (in absence of inhibitors) to which the observed turbidity corresponded.

D-leucine. In all cases the inhibition by D-amino acids on an equimolar basis was in the order D-leucine > D-valine > D-alanine. In no case were

the L forms inhibitory in the range studied. Glycine was more inhibitory than D-alanine.

An evaluation of the variation in extent of inhibition may be obtained from the results presented in Table I, at a common value of 0.050 M of the added D-valine or D-leucine. These results were obtained in a graphically sensitive region of concentration; at 0.100 M all D-valine and D-leucine tubes

TABLE I

*Inhibition of Escherichia coli by D-Valine and D-Leucine at 0.050 M Concentration*

Experiment No.	Growth ratio	
	D-Valine	D-Leucine
	per cent	per cent
4	33	21
5	49	37
6	43	30
7	33	14

TABLE II

*Effect of Pyridoxine and Alanine on Inhibition of Lactobacillus arabinosus Caused by Glycine, D-Valine, and D-Leucine*

Addition to basal medium (2.50 ml.) in tube	Average, 0.100 N acid produced
	ml.
None.....	2.39 ± 0.02
15 mg. glycine.....	2.18 ± 0.01
30 " ".....	1.48 ± 0.06
30 " " + 30 mg. L-alanine.....	2.56 ± 0.05
30 " " + 30 " D-alanine.....	2.69 ± 0.04
30 " " + 0.5 mg. pyridoxine.....	2.35 ± 0.03
30 " D-leucine.....	1.17 ± 0.07
30 " " + 30 mg. L-alanine.....	1.25 ± 0.02
30 " " + 0.5 mg. pyridoxine.....	1.40 ± 0.01
30 " D-valine + 30 mg. L-alanine.....	1.30 ± 0.10

were visually clear. The set of four experiments in Table I was preceded by three experiments which showed the same trends but which lacked sufficient points in the curves for interpolation to 0.050 M.

The effect of glycine upon the growth of *Lactobacillus arabinosus*, in combination with other substances, is illustrated in the data in Table II. Table II shows that the inhibition caused by glycine is reversed by either D-alanine or L-alanine, or by pyridoxine. It may be noted that D- and L-alanine were equally effective. The inhibition caused by D-leucine is, however, not reversed by either L-alanine or pyridoxine. The same lack of reversibility is found for the inhibition by D-valine.

In Table III are presented the effects of some of these same substances on *Escherichia coli*. For this species D-alanine is inhibitory, and the inhibition by glycine is not reversed by L-alanine, D-alanine, or pyridoxine.

TABLE III

*Effect of Pyridoxine and Alanine on Inhibition of Escherichia coli Caused by Glycine*

All results were observed in duplicate.

Addition to basal medium in tube	Effect
None.....	Normal visible growth
25 mg. glycine.....	No visible growth
25 " D-alanine.....	" " "
25 " L-alanine.....	Normal visible growth
25 " glycine + 25 mg. D-alanine.....	No visible growth
25 " " + 25 " L-alanine.....	" " "
25 " " + 0.5 mg. pyridoxine.....	" " "

## DISCUSSION

The inhibitory action of some of the D-amino acids upon bacterial growth is an effect which is not limited to the first species studied, *i.e.*, *Lactobacillus arabinosus*. At least two other species of bacteria are so affected, and preliminary evidence exists for retardation of growth of laboratory animals by high levels of D-amino acids. The concentrations of D-amino acids which are necessary for inhibiting bacterial growth are higher than those usually employed for practical use of other antibacterials. On the other hand the relatively low mammalian toxicity of some D-amino acids (14, 15) does not warrant preclusion of investigation of such properties. The conceivable long term detrimental effects which might result from certain D-amino acids, when present in solutions employed in large amounts clinically, require further study. Of related interest is an investigation of the effects of D-amino acids and derivatives upon neoplasms.<sup>1</sup>

The antipodal specificity found for the amino acids in the present studies makes it desirable to consider that some of the bacterial inhibitions which have been reported for DL-amino acids (16, 17) may have been due to the D isomer. On the other hand, in some cases the activity of the racemate is not the average of the activities of the optical isomers. DL-Valine, for instance, had almost the same activity against *Lactobacillus arabinosus* as had D-valine (2). Analogous behavior has been reported for substrates of proteases (18, 19), but more recent results (20) are at variance with one of these reports (19). In the other case (18), chymotrypsin failed to hydrolyze benzoyl-DL-tyrosylglycylamide, although it hydrolyzed the L isomer. The explanation which has been offered is that the racemate in solution repre-

<sup>1</sup> Fox, S. W., and Wax, H., unpublished experiments.



sents a third substance with distinct properties. The distinct nature of such properties is well illustrated in the solubilities of the various forms of amino acids (21) and in the values for the heats of combustion of DL-leucine as compared to that of either D-leucine or L-leucine (22). With the added fact that L-amino acids possess inhibitory activity in some experiments (23), it appears that conclusions in bacterial experiments can be drawn most safely in comparisons between L and D forms rather than between L and DL molecules.

A similarity in type of inhibition of growth of bacteria by D-amino acids to their effects as substrate residues for proteases has been cited for the belief that D-amino acids may interfere quite directly with the action of "proteosynthetic" enzymes (2). This analogy was based upon the non-hydrolyzability of peptide substrates on one hand (3) and on effects of added unsubstituted D-amino acids (2) on the other. It is therefore of interest that Abderhalden and Abderhalden (4) found that added D-leucine itself greatly decreases the hydrolytic activity of peptidases from a variety of sources. If one accepts the concept that a proteolytic enzyme catalyzes the same reaction in both directions, the accumulated observations substantiate the interpretation that the D-amino acids studied hinder bacterial growth by interfering with proteolytic (= proteosynthetic) enzymes.

The correspondence of the bacterial effects and the substrate effects was close enough to suggest originally such a conclusion (2) for *Lactobacillus arabinosus*. This correspondence held for both isomers of valine, both isomers of leucine, and for L-alanine, but not for D-alanine. In the case of *Escherichia coli*, the correspondence holds for all six forms, since D-alanine is inhibitory to this species. On such a basis, the effect of D-alanine on *L. arabinosus* is anomalous. A clue to such behavior is found in the ability of alanine, especially D-alanine, to reverse the inhibition by glycine. The effect was observed in *Streptococcus faecalis* R by Snell (24) and such behavior has also been found here to hold for *L. arabinosus*. Snell suggested that D-alanine functioned as an intermediate in an inefficient conversion to pyridoxine. Perhaps the important points in the present study are that *E. coli*, which is inhibited by D-alanine, cannot use it in the same way as *L. arabinosus* does (Tables II and III), and that the pattern of inhibition by monoaminomonocarboxylic acids for *E. coli* bears such a close analogy to the effects with peptidase substrates.

Besides the parallelism in the enzymic and bacterial effects of alanine, valine, and leucine, analogous behavior is also seen with glycine. Glycine is found to be inhibitory for the two species of organisms reported here and for others reported previously (8, 25). The glycine residue also exhibits the property of interference with hydrolysis by enzymes (3, 26) as exemplified by "sluggish" peptidolysis of glycylglycine.

The relationship of the D-amino acids to antibiotic D-amino acid derivatives is receiving further attention. The D-amino acid residue has been shown to be one of a number of critical structural features for penicillin through comparison of the activity with the L isomer (27). The corresponding investigation for gramicidin, requiring the preparation of a gramicidin type molecule containing only L residues is not readily feasible by present synthetic methods. A number of molecules in which the D-amino acid residue is repeated do not, however, have high antibacterial activity.<sup>2</sup> If the D-amino acid residue is generally critical for antibiotics of the penicillin and gramicidin classes, other structural features must be concurrently critical.

#### SUMMARY

The growth of *Escherichia coli*, like that of *Lactobacillus arabinosus*, is inhibited by added D-amino acids, at levels at which the L forms do not exhibit such an effect. D-Alanine, which is not inhibitory for *Lactobacillus arabinosus*, slows the growth of *E. coli* to a lesser extent than do D-valine or D-leucine. These effects of amino acids on *E. coli* correspond closely to those observed by others for the hydrolyzability of peptides, constructed from these same amino acids, when subjected to the action of peptidases.

High levels of added glycine inhibit both *Lactobacillus arabinosus* and *Escherichia coli*. In the case of the former species, this inhibition is counteracted by added D-alanine or pyridoxine, whereas such addition to the culture medium of *Escherichia coli* fails to counteract these effects.

The theoretical implications of the results reported are discussed.

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# FLUOROPHOTOMETRIC METHOD FOR THE ESTIMATION OF SALICYLATE IN BLOOD\*

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While salicylates are among the most frequently prescribed drugs, methods for their determination in blood have been neither sufficiently sensitive nor precise. Brodie, Udenfriend, and Coburn (1) have devised a procedure suitable for the clinical measurement of blood salicylate levels if large doses are given. In their method the salicylate is extracted from acidified plasma with ethylene dichloride. A portion of the ethylene dichloride is removed and shaken with an aqueous ferric nitrate solution to produce a colored iron complex. Coburn (2) advises the use of quantities of plasma larger than 1 ml. if the salicyl "radical" is less than 100  $\gamma$  per ml. Peters (3, 4) has drawn attention to the low sensitivity of the method. Volterra and Jacobs (5) applied the xanthoproteic reaction to trichloroacetic acid filtrates for the determination of salicylates. However, their serum blanks in normal subjects were relatively large, corresponding to 3 mg. per cent.

In the method to be presented a simple physical phenomenon, the bluish violet fluorescence of the salicylate ion on exposure to ultraviolet light, will be used for its measurement. Under suitable conditions a high degree of specificity and sensitivity can be attained.

*Principle*—Salicylates are quantitatively separated from proteins by precipitation of the latter with a dilute tungstic acid reagent. Strong alkali is added to increase the fluorescence of the salicylate ion about 9-fold. The blank fluorescence of plasma without salicylate is negligible. The fluorescence is measured directly in a fluorophotometer with the same filters as in the vitamin B<sub>1</sub> determination.

## *Reagents*—

1. Standard salicylate solution (equivalent to 100 mg. of salicylic acid per 100 ml.). 116 mg. of sodium salicylate are dissolved in exactly 100 ml. of water. Working standards are prepared by dilution with water. Store in the refrigerator.

2. 19 per cent HCl.

3. Ethylene dichloride. Do not pipette.

4. 40 per cent NaOH.

5. Dilute tungstic acid reagent. 10 per cent sodium tungstate, 1 volume,

\* Assisted by a fellowship from the Emanuel Libman Fellowship Fund.

mixed with 8 volumes of N/12 sulfuric acid. This solution must be prepared fresh every 2 weeks.

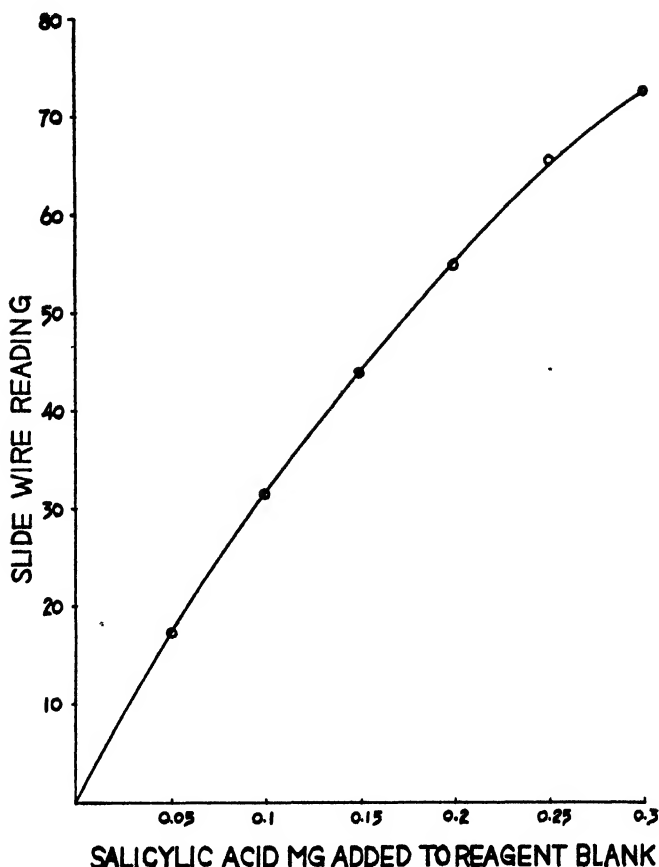


FIG. 1. Standard curve of salicylate fluorescence. Salicylate added to reagent blank mixture.

#### *Procedure 1; Clinical Method*

To 1 ml. of oxalated or citrated plasma (not hemolytic) in a test-tube, add slowly, with shaking, 9 ml. of tungstic acid reagent. After 10 minutes filter. Pipette 5 ml. of filtrate into another test-tube and add 7 ml. of 40 per cent NaOH. Mix. The reagent blank reference solution contains 5 ml. of tungstic acid and 7 ml. of NaOH. Place the solutions in a fluorophotometer for measurement within 30 minutes after adding the alkali.

Fluorophotometric measurement was performed with an instrument con-

taining a balance photocell and bridge circuit (Lumetron<sup>1</sup>). The same filters as in the vitamin B<sub>1</sub> determination are used (maximum transmission of primary 3700 Å, of secondary 4600 Å). The sensitivity of the galvanometer (Rubicon Company) was 0.0025 milliamperes per millimeter. The standard (1 ml. of the standard salicylate solution plus 11 ml. of water) is set at a slide wire reading of 30. The reagent blank is set at 0 with the zero suppressor control. The entire 12 ml. are added to the sample holder (15 ml. capacity) for measurement. Slide wire readings are taken after a 2 minute exposure to the ultraviolet light. The values are then read off a standard curve and multiplied by 2 to correct for dilution.

The standard reference curve for the clinical method (Fig. 1) is prepared by adding varying amounts of standard salicylate solution to the reagent blank mixture. With this procedure the blank reading of plasma without salicylate is of the order of 1 mg. per cent of salicylic acid.

#### *Procedure 2; Modification of Ethylene Method (1)*

To 1 ml. of plasma in a 50 ml. separatory funnel add 0.2 ml. of 19 per cent HCl and mix. After 10 minutes, 10 ml. of ethylene dichloride are added from a graduated cylinder and the separatory funnels are shaken for 3 minutes. The lower layer is transferred to a glass-stoppered test-tube in which 5 ml. of 40 per cent NaOH had previously been placed. Shake for 1 minute. Separate by centrifuging for 5 minutes at moderate speed. Plunge the pipette through the upper layer and draw off 4 ml. of the aqueous phase into a test-tube. Add 8 ml. of water, mix, and measure the fluorescence as above. A reagent blank is run simultaneously. The standard curve is made by subjecting varying amounts of standard salicylate solution to the same procedure. The blank on plasma not containing salicylate is zero.

#### EXPERIMENTAL

The fluorescence of salicylate varies with the pH of the medium. Salicylic acid in distilled water does not fluoresce, whereas sodium or lithium salicylate in distilled water exhibits a bright bluish violet fluorescence on exposure to ultraviolet light. When the salicylates were investigated as fluorescent pH indicators, it was recorded that the fluorescence appears at a pH of 2.5 (6) or 3.0 (7). A sensitive galvanometer can detect fluorescence at even lower pH values. In ultraviolet light a neutralized aqueous solution of sulfosalicylic acid has the identical bluish violet fluorescence. Sali-

<sup>1</sup> Photovolt Corporation, New York.

cylic acid<sup>2</sup> in distilled water does not fluoresce. If alkali is added to a salicylic acid solution, the fluorescence appears at a pH of about 6.3.

The following experiment shows the intensification of fluorescence by alkali. The same concentration of salicylate was used (1.16 mg. of sodium salicylate in 12 ml. of water). As the concentration of alkali was increased to 10 N, the fluorescence rose to twice the reading in distilled water with  $\text{NH}_4\text{OH}$  and nine times when  $\text{NaOH}$  was used. In Fig. 2 the increase in fluorescence was plotted against concentration of  $\text{NaOH}$  on a semilog graph.

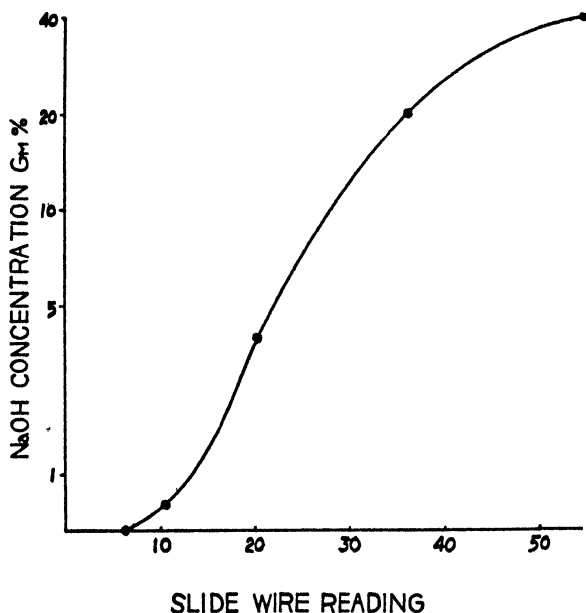


FIG. 2. Increase of fluorescence of salicylate with alkali

With the increase in alkali concentration the fluorescence increased markedly, forming an S-shaped curve.

In strong alkali the fluorescence of salicylate deteriorates slightly during continuous exposure to ultraviolet light. No decay of fluorescence was noted at high pH in the absence of ultraviolet light. Highly reproducible readings can be made if one exposes the solution to be examined to ultraviolet light for a definite period of time. 2 minutes of irradiation with ultraviolet light were found sufficient to allow for the decrease in fluorescence of the plasma blank to a negligible quantity.

The data in Table I indicate that varying quantities of sodium salicylate

<sup>2</sup> Kindly supplied by Dr. Bernard B. Brodie, New York University College of Medicine, New York.

added to 1 ml. of plasma are recoverable with satisfactory precision. The values given are expressed as salicylic acid in accord with routine usage. By this method the drug content of the plasma was recovered with accuracy down to 2 mg. per cent of salicylic acid.

Analyses run on plasma samples of salicylates over a period of a week gave highly reproducible results. It may be concluded, therefore, that the salicylate fluorescence is unchanged if the plasma is stored in the refrigerator.

TABLE I  
*Recovery of Sodium Salicylate Added to Plasma\**

Added	Found	Added	Found
mg.	mg.	mg.	mg.
0.02	0.02	0.30	0.29
	0.02		0.30
	0.02		0.30
	0.02		0.31
0.05	0.04		0.31
	0.05	0.40	0.39
	0.05		0.39
	0.05		0.40
0.10	0.10		0.40
	0.10		0.41
	0.10	0.50	0.49
	0.10		0.52
	0.11		0.53
0.20	0.19		
	0.19		
	0.20		
	0.20		
	0.20		

\* Values expressed as salicylic acid.

#### DISCUSSION

The fluorometric method is more sensitive than the colorimetric, quantities of salicylate as low as 1 to 2 mg. per cent being detectable. Such measurements are without significance if the plasma blank is not zero. In the ethylene dichloride procedure a zero plasma blank is obtained. The direct method on protein-free filtrates is applicable to measurement of low concentrations if the plasma blank determined prior to the administration of salicylate is known. In ordinary clinical usage with the latter procedure the plasma blank was found to be 1 mg. per cent or less. The latter method has the advantage of reference standards made up directly, whereas with the ethylene dichloride the extraction of standards is necessary.

In urine the "salicyl" is present either as free salicylic acid or as some con-



jugated product (salicyluric acid or glucuronide (8)). By use of both ethylene dichloride and carbon tetrachloride methods, Brodie *et al.* (1) demonstrated the absence of appreciable amounts of salicyluric acid in a series of plasma samples from patients on sodium salicylate therapy. By the direct method presented in this communication total salicylate (both free and conjugated) would be measured, whereas free salicylic acid alone is estimated by the ethylene methods.

#### SUMMARY

Solutions of salicylate fluoresce a bright bluish violet under ultraviolet light. The fluorescence is intensified by addition of alkali. A simple procedure for direct determination of salicylates in protein-free blood filtrates is described. The fluorescent method offers rapidity and greater sensitivity than previous methods.

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# STUDY OF A NEW STIMULATORY GROWTH FACTOR

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During the investigation of the folic acid content of natural substances, it was found that aqueous extracts of malt sprouts when assayed with *Streptococcus lactis* R yielded abnormally high values for this vitamin. Further studies proved that the effect is produced by a growth factor unidentifiable with any of the known vitamins, amino acids, strepogenin, or glutamine. It was stimulatory for *S. lactis* R and *Lactobacillus casei*. The presence of a growth factor for *S. lactis* R in malt sprouts was reported by Ruegamer *et al.* (1). Unlike the *S. lactis* R factor of Keresztesy, Rickes, and Stokes (2) the malt sprout factor appears to be equally effective for *S. lactis* R and *Lactobacillus casei*.

The malt sprout factor is not essential for the growth of these organisms even for short periods of incubation except when very small inocula are used. On the other hand, heavier growth results on addition of malt sprout factor with the usual inoculum regardless of whether strepogenin, excess amino acids, or excess vitamins are present in the media used in the investigation.

## EXPERIMENTAL

*Preparation of Extracts*—20 gm. of dried malt sprouts<sup>1</sup> are extracted with 200 ml. of distilled water in a Waring blender for 10 minutes. The extract is filtered through paper and some fine suspended matter is separated by centrifuging at high speed. The supernatant contains the malt sprout factor and may be stored in the refrigerator for months without loss of activity.

*Method of Assay with Streptococcus lactis R*—Varying volumes of the extract are placed in a series of ten tubes and distilled water added to a final volume of 5 ml. To each tube are added 5 ml. of the broth described in Table I. The tubes are shaken and autoclaved 5 minutes at 120°. After cooling, the tubes are inoculated with 1 drop of the suspension prepared as described below. The tubes are shaken and incubated at 30°. Appropriate controls are included. After incubating 16 to 18 hours, the turbidity of each tube is read in the Klett-Summerson colorimeter with the red filter. Table II illustrates typical results obtained with malt sprout

<sup>1</sup> Supplied by Viteena Feed Mills, Inc., St. Louis.

factor extract when either enzyme digest of casein or acid-hydrolyzed casein was used as the amino acid source with the same basal medium.

TABLE I  
*Composition of Media (Double Concentration)*

Vitamin - free acid - hydrolyzed casein or enzyme digest of casein	1.0 gm.	Adenine sulfate	2 mg.
K <sub>2</sub> HPO <sub>4</sub>	0.5 "	Guanine hydrochloride	2 "
Sodium citrate	5.0 "	Xanthine	2 "
Glucose	4.0 "	Uracil	2 "
L-Cystine	40 mg.	Folic acid (Lederle)	10 γ
DL-Tryptophan	40 "	Thiamine hydrochloride	40 "
Asparagine	20 "	Riboflavin	40 "
DL-Alanine	40 "	Nicotinic acid	120 "
Salt Solution B*	1 ml.	Calcium pantothenate	80 "
		Pyridoxine hydrochloride	240 "
		p-Aminobenzoic acid	2 "
		Biotin	0.08 "
		Glass-distilled water	100 ml.
		Adjust pH to 6.8	

\* Salt Solution B contains NaCl 0.5 gm., MgSO<sub>4</sub>·7H<sub>2</sub>O 10 gm., FeSO<sub>4</sub>·7H<sub>2</sub>O 0.5 gm., MnSO<sub>4</sub>·2H<sub>2</sub>O 0.337 gm., glass-distilled water 250 ml.

TABLE II  
*Growth Response of Streptococcus lactis R to Increasing Quantities of Malt Sprout Factor*

Malt sprout factor added per tube	Turbidity*	
	Source of amino acids	
	Acid-hydrolyzed casein	Enzyme digest of casein
ml.		
0.005	77	147
0.01	103	138
0.03	151	162
0.05	172	170
0.08	192	195
0.10	198	210
0.50	218	244
1.0	238	272
None	40	122

\* Turbidity measured on a Klett-Summerson colorimeter.

*Preparation of Inoculum*—Stock cultures of *Streptococcus lactis* R are carried in stabs made by adding 1.5 per cent agar to the broth in Table I, diluted with an equal volume of water and containing 1 mg. of Wilson's liver fraction L<sup>2</sup> for each 10 ml. Daily transfers are made to broth of the

<sup>2</sup> Liver fraction L produced by The Wilson Laboratories, Chicago.

same composition with the liver fraction added. After incubating 18 to 20 hours at 30°, the culture is centrifuged and washed three times with 10 ml. of normal saline. After the third washing, the organisms are suspended and diluted with normal saline to give a reading of 30 to 35 in the Klett-

TABLE III  
Growth Response Produced by Addition of Various Supplements to  
Media with and without Malt Sprout Factor

Supplement added	Supplement per tube	Malt sprout factor per tube	Turbidity*	
			Amino acid source	
			Acid-hydro- lyzed casein	Enzyme digest of casein
No addition made	mg.	ml.		
	0	0	60	122
	0	1.0	211	228
	5.0	0	145	173
Wilson liver fraction L	5.0	1.0	222	238
	4.0	0	30	77
Amino acid mixture†	4.0	1.0	205	236
	10.0	0	8	28
	10.0	1.0	170	142
	ml.			
Vitamin mixture‡	1.0	0	61	117
	1.0	1.0	195	236
	2.5	0	49	68
	2.5	1.0	170	219
Enzyme digest of casein	mg.			
	100	0	110	141
	100	1.0	243	264
	200	0	109	138
	200	1.0	226	247

\* Inoculum = *Streptococcus lactis* R as described above. Turbidity read on Klett-Summerson colorimeter.

† Contains 2 mg. of each of all the known amino acids per ml.

‡ The vitamin mixture contains thiamine hydrochloride 2 γ, niacin 6 γ, *p*-aminobenzoic acid 0.1 γ, riboflavin 2 γ, pyridoxine hydrochloride 12 γ, pyridoxal hydrochloride 6 γ, biotin 0.004 γ, calcium pantothenate 4 γ.

Summerson colorimeter. The final inoculum is prepared by diluting this suspension 1000 times with normal saline.

Before any conclusion could be reached, it was necessary to eliminate the possibility that this stimulation might be due to streptogenin, amino acids, or vitamins. Two media were used for the study, having identical composition (Table I) with the exception of the amino acid source. One medium contained 50 mg. of enzyme digest of casein and the other 50 mg.

of acid-hydrolyzed casein per 10 ml. Supplements of the substances tested were made to both media. For the source of streptogenin Wilson's liver fraction L and enzyme digest of casein were employed. A stock solution of all the amino acids and one of all of the known vitamins were added, as indicated in Table III, in addition to the quantities already present in the medium. The results are summarized in Table III. The values obtained with the enzyme digest of casein are higher than those with acid-hydrolyzed casein, owing probably to streptogenin activity. In every instance, while the addition of supplements did not materially affect the growth response over that obtained in control tubes, the addition of 1 ml. of malt sprout factor produced a marked response. It is interesting to note that there is inhibition of growth with increasing amounts of vitamins or amino acids in crystalline DL form. No such reaction occurs when amino acids are increased as in the enzyme digest of casein. Even with this inhibitory effect malt sprout factor produces a pronounced growth stimulation.

TABLE IV  
*Heat Stability of Malt Sprout Factor; Klett Readings*

Malt sprout factor per tube	Autoclaved 5 minutes at 120°	Sintered glass filter
ml.		
0.5	106	120
1.0	170	168

#### DISCUSSION

Thus malt sprout factor appears to contain a factor, or factors, which elicits a growth response not obtained by any of the known growth factors listed in Table III. A cursory examination of other sources for this factor reveals that it is present in aqueous extracts of whole fresh liver, barley leaf meal, and fresh tomato juice. It is not present in pernicious anemia liver concentrates, whey, horse serum, or Wilson's liver fraction L.

Malt sprout factor is differentiated from the factor of Keresztesy, Rickes, and Stokes (2), since it appears to have approximately the same activity for *Lactobacillus casei* as it does for *Streptococcus lactis*. They also mention that folic acid can replace their factor for all bacteria which utilize the latter. Folic acid does not produce the response obtained with malt sprout factor. As demonstrated in Table IV, malt sprout factor is stable to autoclaving and, since it is not present in liver concentrates, it appears to differ from the factor discussed by Ruegamer *et al.* (1). Hall (3) reported the replacement of folic acid by histidine and its effect on *Streptococcus lactis*. Since the broth used in the present paper contained added histidine and gave no apparent response, malt sprout factor cannot be identified with it.

It is apparent that this factor can interfere with microbiological assays in which the incubation period is short. This discrepancy is most evident when large quantities of the material to be tested are used. The data on the effect of this factor over longer periods of incubation are not complete at this time.

With work now in progress to purify this factor, or factors, it is hoped that a more thorough examination of the effect of this stimulation in microbiological assays can be made.

#### SUMMARY

A stimulatory factor in malt sprouts effective for *Lactobacillus casei* and *Streptococcus lactis* R has been examined and found to differ from known vitamins, amino acids, streptogenin, and other growth factors. It is present in other natural substances and is heat-stable.

The possible effect of this stimulatory factor in microbiological assays is discussed.

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# A NOTE ON THE APPLICATION OF THE COLORIMETRIC NINHYDRIN DETERMINATION TO STUDIES OF ENZYME KINETICS

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Although the validity of the colorimetric determination of single amino acids with ninhydrin, described by Harding and MacLean (1), is unquestioned, the failure of this determination for mixtures of amino acids, together with the observation that ninhydrin produces a color with ammonia (2) and with certain amines and amides (3), seems to have led to the neglect of this useful procedure. The discovery by Bergmann and his coworkers (4, 5) of simple synthetic substrates for proteolytic enzymes has provided a powerful tool for the study of enzyme kinetics, but the analytical methods commonly used have been the limiting factor, both in time and accuracy, in these studies.

A determination of the absorption spectrum of the color developed with phenylalanine in the Harding-MacLean method with a Coleman junior spectrophotometer revealed a rather sharp maximum at 572  $m\mu$ . Ninhydrin-pyridine-water blanks have a minimum absorption in this same region so that unchanged ninhydrin causes no interference with the determination. Although the color developed by quantities of DL-phenylalanine up to 10 micromoles was found to follow Beer's law when transmissions were measured at 572  $m\mu$ , to date a suitable blank has not been found, with the result that the straight line relating per cent transmission on a semi-logarithmic scale to quantity of phenylalanine does not pass through the point corresponding to zero phenylalanine and 100 per cent transmission. The intersection of the calibration line with the 100 per cent transmission axis occurs at 0.3 micromole of phenylalanine. This value must be regarded as the present lower limit of the method.

Harding and MacLean (1) did not apply the method to quantities of amino acids greater than 3.6 micromoles, but it has been found that the quantities of reagents used by these workers are adequate for amounts of phenylalanine up to 5 micromoles. The determinations were extended to 10 micromoles by simply doubling the quantities of reagents used; *i.e.*, by using 2 ml. of 10 per cent pyridine and 2 ml. of 2 per cent ninhydrin. A series of determinations indicated that quantities of phenylalanine between 0.5 and 10 micromoles could be estimated with an error not greater than



0.06 micromole for the lowest concentration and 0.1 micromole for the highest concentration.

Representative results obtained in following the hydrolysis of carbobenzoxyglycyl-DL-phenylalanine (0.048 M with respect to the L form) by carboxypeptidase ( $4.03 \times 10^{-4}$  mg. of N per ml. of reaction solution) in the presence of 0.0025 M D-phenylalanine are shown in Table I.<sup>1</sup> The enzymatic reaction was run at 25° in 0.04 M phosphate buffer, pH 7.50, which was 0.1 M with respect to LiCl. The blank consisted of substrate and D-phenylalanine, the enzyme contributing no detectable color at this low concentration. The substrate alone at a concentration of 0.05 M gave 99 per cent transmission.<sup>2</sup> 0.2 ml. samples were withdrawn at intervals and

TABLE I  
*Hydrolysis of Carbobenzoxyglycyl-L-phenylalanine by Carboxypeptidase*

Time	Transmission	Concentration of phenylalanine	Hydrolysis	k*	Calculated hydrolysis†
min.	per cent	micromoles per ml.	per cent	$10^{-3} \text{ sec.}^{-1}$	per cent
22	79.8	6.8	14.2	7.01	14.2
42	63.5	12.3	25.6	7.06	25.5
62	52.7	16.8	35.0	6.98	35.1
82	43.5	21.5	44.8	7.24	43.5
102	39.4	23.9	49.8	6.78	50.9
123	33.9	27.5	57.3	6.94	57.6
Mean.....				7.00	

\* Apparent first order reaction constant.

† Calculated from mean value of apparent first order reaction constant.

the color developed was diluted to 100 ml. Transmissions were read in  $10 \times 75$  mm. cuvettes.

This method has been adopted for routine work in this laboratory and has been found to give results in good agreement with, but much more consistent than, those obtained by the ninhydrin determination of free amino acids in the Van Slyke manometric apparatus. The increased sensitivity of the colorimetric method is being utilized in a detailed study of the mech-

<sup>1</sup> The results presented here were obtained by Miss Elaine Elkins of this laboratory in the course of an investigation which will be fully reported in the near future.

<sup>2</sup> This is true only for freshly prepared solutions of this substrate. Prolonged storage of the solution, even in the cold, causes sufficient spontaneous hydrolysis to produce an appreciable color with the present method (equivalent to up to 10 per cent absorption). This introduces a serious error in kinetic studies with carboxypeptidase when carbobenzoxyglycyl-DL-phenylalanine is used as the substrate, since the D-phenylalanine formed by spontaneous hydrolysis may strongly inhibit enzymatic activity (6).

anism of the hydrolysis of carbobenzoxyglycyl-L-phenylalanine by carboxypeptidase.

This work has been supported by grants from the Rockefeller Foundation, from the National Institute of Health, United States Public Health Service, and from the Duke University Research Council.

#### SUMMARY

The utility of the colorimetric amino acid determination of Harding and MacLean in studies of the reaction kinetics of proteolytic enzymes with synthetic peptides has been demonstrated.

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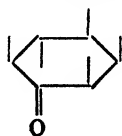
# OXIDATION OF INOSITOL BY ACETOBACTER SUBOXYDANS\*

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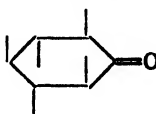
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Streptamine, a degradation product of streptomycin, has been established to be one of the eight possible *meso* forms of 1,3-diamino-2,4,5,6-tetrahydroxycyclohexane (1-3). Consideration of possible synthetic approaches to such a molecule suggested that oxidation products of inositol might serve as useful intermediates. Two monoketo derivatives of inositol (inososes) have been prepared and their stereochemical configuration elucidated by Posternak (4-6). *epi*-Inosose (I) is obtained by the oxidation of inositol with nitric acid (7) and *scyllo*-inosose (II) by the action of *Acetobacter sub-*



(I)

*epi*-Inosose



(II)

*scyllo*-Inosose

*oxydans* on inositol<sup>1</sup> (5, 8). In addition to these two substances a crude "diketo inositol" has been described in a series of papers by Dunning, Ful-

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<sup>1</sup> Two methods of nomenclature for the inososes were considered by Posternak (6). In one the inosose is named on the basis of the two inositols it yields on reduction. Thus Compound I, which yields scyllitol and *meso*-inositol, is called *scyllo-meso*-inosose or *scyllo-ms*-inosose. Compound II is named *dl-epi-meso*-inosose or

mer, Pitcher, Underkofler, and Guymon (9-13). These workers obtained this substance (rather than *scyllo*-inosose) by the action of *A. suboxydans* (American Type Culture Collection No. 621) on inositol. If the ketone groups in this diketo inositol are in the 1,3 position, it might afford promise as an intermediate in the synthesis of streptamine. We therefore undertook a study of the fermentation of inositol by *A. suboxydans* in the hope of obtaining the diketone.

The fermentation of inositol was carried out on a 3 per cent inositol-0.1 per cent sorbitol medium at 30° for a 14 day period with *Acetobacter suboxydans* 621 obtained from the American Type Culture Collection. Every effort was made to duplicate the conditions of Dunning (10) exactly in so far as this is possible in such experiments. The fermentation mixture was clarified with lead acetate, and excess lead was removed over an exchange resin column rather than with hydrogen sulfide. By this minor alteration the development of a brown color was avoided. The final product was obtained as a white crystalline solid which could be fractionated from water or aqueous methanol. The decomposition points of successive fractions usually declined from about 198° to about 174°. (Pure *scyllo*-inosose melts with decomposition at 200-202° (5); diketo inositol melts in the range 184-195° (13).) Since a pure compound was not obtained by extensive recrystallization of these fractions, attention was turned to the possibility of effecting separation through various derivatives. The crude fractions were treated with phenylhydrazine under the conditions described by Posternak (5). A heavy red precipitate slowly formed at room temperature and increased in amount over a 2 day period. Extraction of the crude product with ether removed a red pigment. From the residue a yellow crystalline compound was separated by chromatography over an alumina column. This compound was characterized as an osazone<sup>2</sup> by analyses and absorption

*dl-epi-ms*-inosose, since it yields *dl-epi*-inositol and *meso*-inositol on reduction. In this case the term *dl-epi*-inosose is preferable to avoid the inconsistency of applying the term *meso* to a racemic form. Likewise Compound I is usually designated simply as *scyllo*-inosose. In the second system each configuration is represented by a fraction in which the position numbers of the hydroxyl groups lying "above" the plane of the ring form the numerator and the numbers of those lying below the plane form the denominator. Thus *scyllo*-inosose is named inosose- $\frac{246}{35}$  or for convenience

inosose-(246/35). This system is applicable to all inositol derivatives whose configuration is known. The first method, employing trivial names, is less general and runs into obvious inconsistencies. However, since the terms *epi*- and *scyllo*-inosose have been established by usage, they will be employed in this paper.

<sup>2</sup> Posternak (7) prepared an osazone (m.p. 192-194°) from the hypobromite oxidation product of inositol. This may be identical with our osazone. Chargaff and Magasanik (14) recently reported an osazone (m.p. 213-214°) from the oxidation

spectrum. It was discovered subsequently that the crude phenylhydrazine reaction product contained both a phenylhydrazone and an osazone. The phenylhydrazone could be separated from the mixture by extraction with methanol. The residue yielded pure osazone on recrystallization from pyridine-water.

The interpretation of these results is complicated by the observation that pure *scyllo*-inosose and inosose phenylhydrazone also yield the osazone on extended treatment with phenylhydrazine. Furthermore it was discovered that when the phenylhydrazine reaction mixture was cooled, stirred vigorously, and the walls of the flask scratched to induce rapid crystallization, the product consisted mainly of phenylhydrazone. In this way good yields of the phenylhydrazone of *scyllo*-inosose were obtained from each of the fractions.

These data indicate that a considerable proportion of the osazone previously obtained resulted from the oxidizing action of phenylhydrazine and that the major portion of the fermentation product consisted of *scyllo*-inosose.

Three other strains of *Acetobacter suboxydans*,<sup>3</sup> strains NRRL-B-72, B-474, and B-487, were also studied under the conditions of Dunning (10). The first two gave results essentially identical with those obtained with the American Type Culture Collection No. 621. The third strain, B-487, metabolized inositol very slowly, and even after 10 to 12 days more than 50 per cent remained unattacked.

Since the possibility of oxidation by phenylhydrazine introduced a complicating factor, it was decided to prepare acetyl derivatives of the various fractions. For this purpose a large fermentation with strain B-474 was processed and the crude product separated into five fractions from water and aqueous methanol. Phenylhydrazones and acetyl derivatives were prepared. In addition each fraction was converted to the oxime. This derivative is obtained in good yield on treating pure *scyllo*-inosose with hydroxylamine at pH 13 to 14. The sodium salt of the oxime separates as a nicely crystalline product with a sharp decomposition point. The free oxime is more soluble and less stable than the sodium salt and is not a useful derivative.

The results of this study are summarized in Table I. All the fractions gave the phenylhydrazone of *scyllo*-inosose in good yield. Fraction I, how-

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product of *l*-inositol by *Acetobacter suboxydans*. The substance may be the *l* anti-pode of our *dl*-osazone.

<sup>3</sup> The authors wish to express their appreciation to Dr. William C. Haynes of the Northern Regional Research Laboratory, Peoria, Illinois, who generously supplied the cultures of strains NRRL-B-72, B-474, and B-487.

ever, gave a gummy acetyl derivative which resisted all attempts at crystallization. Nevertheless, this fraction gave a good yield of phenylhydrazone, and reconversion of the latter to inosose gave a product which was readily converted to crystalline pentaacetyl-*scyllo*-inosose. The over-all yield in these steps was 70 per cent. All other fractions gave good yields of the pentaacetyl derivative directly.

Variation of fermentation time was studied next. As a basis for this work the rate of disappearance of inositol in control flasks was determined by a yeast assay method. Somewhat to our surprise the oxidation of the inositol was complete in 3 to 4 days. (Previous workers employed 12 to 20 day fermentation periods but did not determine the rate of disappearance of inositol.) Fermentation products were processed after various intervals

TABLE I  
*Derivatives from Fractions of Strain NRRL-B-474 Inositol Fermentation Product*

*Yields are based on 1.0 gm. samples of each fraction.*

Fraction No. and total weight	Pentaacetate		Phenylhydrazone		Oxime sodium salt	
	Weight of product	Yield	Weight of product	Yield	Weight of product	Yield
	gm.	per cent	gm.	per cent	gm.	per cent
I (17.23 gm.)	See text		1.08	72	0.90	74
II (57.71 gm.)	1.56	72	1.02	68	0.84	70
III (6.7 gm.)	1.66	76	0.98	65	0.94	78
IV (4.00 gm.)			0.93	62		
V (3.75 gm.)			0.86	57		
Inosose (pure)		81		75		

ranging from 4 to 14 days. A very high yield of crude inosose was obtained from the 4 day fermentation mixture. This material, without purification, gave excellent yields of pentaacetyl derivative and of phenylhydrazone. The products from longer fermentations generally gave slightly lower yields of the inosose derivatives. In the phenylhydrazine reaction more of the red pigment was produced. (Pure inosose gives only a slight amount of colored material.) Certain fractions failed to yield a crystalline pentaacetyl derivative until they had been purified through the phenylhydrazone. It seems likely that *scyllo*-inosose is first formed and then undergoes very slow further reaction on standing. In this connection it might be noted that inosose is slowly oxidized by air, especially at an alkaline pH (15). The nature of the contaminant in the older fermentation products has not been determined. Kluyver *et al.* (15) have suggested that the oxidation of inositol by *Acetobacter suboxydans* may proceed through hexahydroxybenzene (enol

form of triketoinositol). We have no direct evidence for its presence in our fermentation mixtures, except for the suggestive observation that hexahydroxybenzene gives a red pigment with phenylhydrazine.

In order to obtain additional quantitative data on the fermentation, the oxygen uptake was determined manometrically on a series of flasks. At the end of 3 days oxygen consumption ceased (at this point only a trace of inositol remained) and the total uptake ranged from 0.52 to 0.57 mole of oxygen per mole of inositol.

These data clearly show that under the conditions of our experiments the major portion of the inositol fermentation product is *scyllo*-inosose. All strains of *Acetobacter suboxydans* tested gave similar products and no evidence was obtained for the presence of an appreciable amount of diketo inositol in the fermentation mixtures.

#### EXPERIMENTAL

*Fermentation of Inositol*—Stock cultures of *Acetobacter suboxydans* 621 and strains NRRL-B-474, B-72, and B-487 were maintained on agar slants (1.5 per cent agar, 0.5 per cent Difco yeast extract, 0.5 per cent sorbitol). The first two strains were used for most of our experiments. A sterile sorbitol medium (10 per cent sorbitol, 0.5 per cent Difco yeast extract) was inoculated with stock culture and incubated for 3 days. A 10 ml. subculture was used to inoculate 200 ml. of unbuffered inositol medium (3 per cent anhydrous *meso*-inositol, 0.1 per cent sorbitol, 0.5 per cent Difco yeast extract). The culture was incubated in a 2 liter Erlenmeyer flask at 30° for periods varying from 4 to 14 days. Later it was discovered that the oxidation proceeded smoothly at room temperature (26–28°) and several large scale runs were made in 2 quart milk bottles (each containing 200 ml. of medium) stacked horizontally on the desk top. The progress of the fermentation was followed by microbiological assays for inositol. Strain NRRL-B-487 oxidized inositol slowly and incompletely. Even after 10 days the medium assayed 19.6 mg. of inositol per ml. (original concentration, 30 mg. per ml.). The remaining three cultures oxidized inositol rapidly and exhibited no important differences in their behavior. After 3 days each of these three fermentation mixtures assayed 0.5 to 1.0 mg. of inositol per ml.

*Microbiological Assay for Inositol*—The pyridoxine assay method of Atkin *et al.* (16) was modified for use in assaying inositol. (Pyridoxine was supplied in the medium.) The test organism, *Saccharomyces carlsbergensis* (No. 26,803),<sup>4</sup> gave a reproducible growth curve at levels of 0 to 15  $\gamma$  of inositol per 10 ml. after incubation with shaking for 16 to 18 hours. Growth

<sup>4</sup> The culture of *Saccharomyces carlsbergensis* was kindly supplied by Dr. O. C. Bird of Parke, Davis and Company.



was determined by measuring turbidity with an Evelyn colorimeter and the samples were calculated by reference to the standard curve.

*Isolation and Fractionation of Fermentation Product, Acetobacter suboxydans 621*—The contents of ten 14 day fermentation flasks (American Type Culture Collection No. 621) were combined (2200 ml.) and treated with 16.5 ml. (0.75 per cent by volume) of saturated lead acetate solution. The mixture was filtered with the aid of Filter-Cel and the filtrate was passed through a  $4.5 \times 16$  cm. Amberlite IR-100 column in the acid phase to remove excess lead ions. The column was washed with 300 ml. of water. The lead-free solution was concentrated to a small volume under reduced pressure and the precipitate was removed by filtration, giving 46 gm. of material decomposing at  $192-194^{\circ}$ .<sup>5</sup> An equal volume of alcohol was added to the filtrate, giving 7.5 gm. of material decomposing at  $178-184^{\circ}$ . The filtrate on concentration gave a dark gum which was discarded. 25 gm. of the first fraction were recrystallized from hot water and additional crops were precipitated from the filtrate by concentration and addition of methanol. The following fractions were obtained: Fraction A 2.75 gm. (decomposition  $195-198^{\circ}$ ), Fraction B 14.7 gm. (decomposition  $184-187^{\circ}$ ), Fraction C 3.5 gm. (decomposition  $179-183^{\circ}$ ), Fraction D 1.6 gm. (decomposition  $177-179^{\circ}$ ). Extensive fractionation was carried out on these products and on similar ones from other fermentations, with water, aqueous methanol, and aqueous ethanol as the solvents. None of these fractions appeared to be pure, although the results of later experiments indicated that all of them consisted to a very large extent of *scyllo*-inosose.

*Reaction of Crude Fermentation Product with Phenylhydrazine*—13.5 gm. of Fraction B were dissolved in 360 ml. of water at  $60^{\circ}$  and the solution was filtered and cooled to room temperature. 18 ml. of redistilled phenylhydrazine and 36 ml. of glacial acetic acid were added and the solution was allowed to stand at room temperature for 2 days. The dark precipitate was filtered and air-dried, giving 8.0 gm. of a dark brownish red product. The filtrate deposited more precipitate on standing and after 2 days a second crop of 3.4 gm. was removed. 10 ml. of phenylhydrazine and 20 ml. of glacial acetic acid were added to the filtrate and after 1 day 2.5 gm. of precipitate were obtained. The dried solids were extracted with ether which removed a dark red pigment. The ether-extracted residues from the three fractions were light tan solids weighing 5.8, 1.7, and 1.5 gm., respectively (total 9.0 gm.).

In the purification of these and similar products chromatography was employed. Alumina was poured into the column as a slurry in methanol and washed thoroughly with methanol. The crude product was dissolved

<sup>5</sup> The melting and decomposition points reported in this paper were determined on the K  f  r micro block unless otherwise specified.

in the minimum amount of pyridine and applied to the column, which was then developed with absolute methanol and pyridine-methanol. The latter solvent markedly increased the rate of development. A red band came through the column first, followed by a yellow band. A dark red pigment remained adsorbed at the top of the alumina column. The yellow band consisted of *dl*-scyllo-inosose osazone.

The first two products above (5.8 gm. + 1.7 gm.) were dissolved in pyridine, applied to a  $4.5 \times 18$  cm. alumina column, and developed with methanol and pyridine-methanol. The first 240 ml. of the percolate contained 529 mg. of a red gummy solid which was almost entirely soluble in ether. The next five fractions (1900 ml.) were combined and concentrated, giving 6.16 gm. of a yellow solid. This material was very soluble in pyridine; the addition of water or methanol caused crystallization of yellow needles decomposing at  $198-200^\circ$ . This material gave the correct analytical data for *scyllo*-inosose osazone and an ultraviolet absorption spectrum very similar to that of glucosazone.

$C_{18}H_{20}N_4O_4$ (356.38).	Calculated.	C 60.66, H 5.66, N 15.71
	Found.	" 60.93, " 5.67, " 15.67

Ultraviolet absorption maxima: *scyllo*-Inosose osazone,  $\epsilon_{2590} = 19,800$ ,  $\epsilon_{3190} = 10,000$ ,  $\epsilon_{3990} = 16,200$ , glucosazone,  $\epsilon_{2550} = 18,100$ ,  $\epsilon_{3100} = 10,000$ ,  $\epsilon_{3860} = 20,000$ .

*Preparation of scyllo-Inosose Phenylhydrazone from Crude Fermentation Product*—13 gm. of fermentation product similar to that used in the preceding section were dissolved in 360 ml. of hot water. The solution was cooled to room temperature and 18 ml. of phenylhydrazine and 36 ml. of glacial acetic acid were added. The flask was immediately chilled in an ice bath and the walls were scratched with a glass rod until precipitation of the phenylhydrazone occurred. After 30 minutes the red precipitate was filtered, dried, and washed with ether, giving 16 gm. of a pink crystalline phenylhydrazone melting with decomposition at  $167-169^\circ$ . Recrystallization of this material from methanol gave 12.0 gm. (61 per cent yield) of colorless phenylhydrazone melting with decomposition at  $176-177^\circ$ . Posternak reported that *scyllo*-inosose phenylhydrazone decomposes at  $184^\circ$  when heated rapidly in a capillary tube.

The filtrate from the phenylhydrazone deposited additional precipitate on standing. This yielded a small quantity of osazone on extraction with ether and recrystallization of the residue from pyridine-water or pyridine-methanol.

*Preparation of scyllo-Inosose from Phenylhydrazone*—The conversion of phenylhydrazone to inosose was effected by a slight modification of the pro-

cedure of Posternak (5). 8 gm. of the phenylhydrazone were mixed with 80 ml. of ethanol, 8 ml. of benzaldehyde, and 4 ml. of glacial acetic acid; then 400 ml. of boiling water were added. The mixture was refluxed for 15 minutes and was then cooled and extracted with ether to remove the benzaldehyde phenylhydrazone. The aqueous layer was concentrated to 50 ml., and 50 ml. of methanol were added. After standing overnight in the ice box the crystalline precipitate was removed, washed with methanol, and air-dried, giving 4.7 gm. (88 per cent yield) of *scyllo*-inosose. The decomposition point of *scyllo*-inosose varies with the rate of heating. On a Köffler block with rapid heating, melting with decomposition occurs at 199–202°, with slow heating at 194–196°. In either case browning occurs before the melting point is reached. Posternak reported that *scyllo*-inosose melted at 205–208° when heated rapidly in a capillary tube (5).

*Preparation of Derivatives from Pure scyllo-Inosose.* 1. *Phenylhydrazone*—The reaction of inosose or of the crude fermentation product with phenylhydrazine was carried out by a modification of the conditions of Posternak (5).

1 gm. of pure *scyllo*-inosose was dissolved in 30 ml. of warm water. The solution was cooled to room temperature and 3.0 ml. of glacial acetic acid and 1.5 ml. of phenylhydrazine were added. The flask was cooled immediately in an ice bath and vigorously scratched to hasten crystallization. After 30 minutes the pink precipitate was filtered, washed with cold water, and air-dried, giving 1.36 gm. of colored phenylhydrazone melting with decomposition at 175–177°. The material was washed with ether and recrystallized from 40 ml. of hot methanol, giving 1.12 gm. of colorless needles (75 per cent yield) melting with decomposition at 176–177°.

A second 1 gm. quantity of pure *scyllo*-inosose was treated as above, except that the flask was allowed to stand undisturbed for 2 days at room temperature. The precipitate was filtered and dried, giving 1.25 gm. of material. However, on recrystallization only a part of this material dissolved in methanol and from the solution only 0.67 gm. (45 per cent yield) of phenylhydrazone was obtained. The residue (0.5 gm.) was a yellow solid which yielded 0.3 gm. of osazone on recrystallization from pyridine-water.

The osazone is also obtained by treating the preformed phenylhydrazone with phenylhydrazine, although the reaction is slower (probably due to the low solubility of the phenylhydrazone). A suspension of 0.75 gm. of the phenylhydrazone in 15 ml. of water was treated with 0.75 ml. of phenylhydrazine and 1.5 ml. of glacial acetic acid. After 3 days at 25° the deep red precipitate was filtered, air-dried, and extracted with ether, giving 0.35 gm. of tan solid. This was extracted successively with 13 ml. and 5 ml. portions of boiling methanol, leaving 0.17 gm. of residue which was recrystallized from 3 ml. of warm pyridine by adding 25 ml. of water. The precipitate

was filtered, giving 0.15 gm. of *dl*-scyllo-inosose osazone as yellow lustrous needles decomposing at 192–194°. A second recrystallization raised the decomposition point to 194–196°.

2. *Pentaacetyl-scyllo-inosose*—The acetylation was accomplished by the acetic anhydride-sulfuric acid procedure of Posternak (5). 3 gm. of inosose were added to 36 ml. of 1:19 sulfuric acid-acetic anhydride mixture. The reaction mixture was warmed to 50° and allowed to stand at room temperature for 2 hours, during which time crystals separated. The mixture was cooled in an ice bath and filtered. The precipitate was washed with glacial acetic acid and ether, giving 5.30 gm. (81 per cent yield) of pentaacetyl *scyllo*-inosose melting at 209–212°. Recrystallization of this material from glacial acetic acid containing a trace of sulfuric acid gave the pure derivative melting at 211–212°. We confirmed the observation of Posternak (5) that repeated recrystallization of the 212° melting product from glacial acetic acid alone gave a pentaacetyl derivative melting at 145–147°. This was readily reconverted to the 212° form.

Treatment of *scyllo*-inosose or the pentaacetyl derivative with acetic anhydride and sodium acetate caused aromatization to 1,2,3,5-tetraacetoxybenzene, as described by Posternak (5).

3. *Sodium scyllo-Inosose Oximate*—A solution of 15.6 gm. of hydroxylamine hydrochloride and 15.5 gm. of sodium hydroxide in 135 ml. of water was cooled to about 10°, and 26.7 gm. of *scyllo*-inosose were added with stirring. The oxime sodium salt crystallized in a few minutes. After 1 hour, the precipitate was filtered (m.p. 136–137°) and recrystallized by being dissolved in 100 ml. of boiling water and 75 ml. of absolute ethanol added. On cooling there were obtained 29 gm. (90 per cent yield) of colorless prisms melting at 138–139° (capillary) with decomposition.

$C_6H_{10}NO_5Na$  (215.15). Calculated, N 6.51; found, N 6.60

The free oxime was obtained by triturating the sodium salt with 125 ml. of glacial acetic acid. The viscous oil initially formed solidified to a colorless microcrystalline powder. This material was washed twice with absolute ethanol, giving the free oxime decomposing at about 140° (capillary). The oxime is very soluble in water and darkens on standing.

Attempts to prepare the free oxime directly from inosose at a lower pH were not successful. The hexaacetyl derivative was prepared by treating the free oxime with 6 volumes of acetic anhydride containing a trace of sulfuric acid for 1 hour at 100°. The clear solution was cooled and poured into 30 ml. of water. The oil which separated soon crystallized, giving 0.9 gm. of colorless needles. This material was recrystallized from absolute ethanol, giving pure hexaacetyl-*scyllo*-inosose oxime (m.p. 111–112°) (capillary).

$C_{12}H_{22}NO_{12}$  (445.37). Calculated. C 48.54, H 5.20, N 3.15  
Found. " 48.65, " 5.08, " 3.22

*Fractionation and Characterization of Fermentation Product, Strain NRRL-B-474*—Sixteen flasks (96 gm. of inositol) were inoculated with strain NRRL-B-474 and incubated at 30° for 14 days. The fermentation mixtures were combined and deproteinized with 28 ml. of saturated lead acetate solution. The suspension was filtered with the aid of Filter-Cel and excess lead was removed by passing the filtrate through an Amberlite IR-100 column in the acid phase. The column was washed with 300 ml. of water and the combined solutions were concentrated *in vacuo* to 190 ml., causing crystals to separate. The mixture was warmed to 50–60° and filtered. The precipitate was washed with 80 ml. of hot water and air-dried, giving 17.2 gm. of material decomposing at 205° (Fraction I). The combined filtrate and washings were diluted with water to 320 ml., and 800 ml. of methanol were added. After 20 hours at 5° the crystalline precipitate was collected, washed with methanol, and dried, giving 57.5 gm. of material decomposing at 192° (Fraction II). 800 ml. of methanol were added to the filtrate and the mixture was stored in the ice box overnight. The precipitate (Fraction III) weighed 6.7 gm. (decomposition 185°). The filtrate was concentrated to 35 ml. and cooled, giving 4.0 gm. (decomposition 185°) of material (Fraction IV). Addition of 950 ml. of methanol to this filtrate gave 3.8 gm. (decomposition 184°) (Fraction V). The total weight of recovered material was 89.2 gm., which represents a 93 per cent yield calculated as inosose.

In order to characterize the fractions the phenylhydrazone, oxime, and pentaacetyl derivatives were prepared from each under the conditions previously described. The results of this study are summarized in Table I.

Fraction I did not yield a crystalline pentaacetyl derivative directly. To determine the maximum amount of impurity present, 2.0 gm. of Fraction I were converted to the phenylhydrazone (yield 2.65 gm., decomposition 175–176°). From the phenylhydrazone 1.54 gm. of *scyllo*-inosose were obtained and this material gave 3.06 gm. of pentaacetyl-*scyllo*-inosose (m.p. 210–212°). The over-all yield of pure pentaacetyl derivative from Fraction I was 70 per cent. Since pure *scyllo*-inosose in a similar series of reactions did not give an appreciably higher yield, it is obvious that the main component of Fraction I is *scyllo*-inosose.

*Manometric Study of Inositol Fermentation by Acetobacter suboxydans*—2 liter Erlenmeyer flasks containing 200 ml. of inositol oxidation medium were sterilized and inoculated with *Acetobacter suboxydans* 621. The flasks were connected to manometers and a source of oxygen. A control flask, containing all of the materials present in the experimental flasks except

inositol, was also set up. The oxygen uptake in the flasks was determined at intervals and after every reading oxygen was admitted, so that the pressure never deviated greatly from atmospheric. The consumption of oxygen ceased after 72 hours (at this point only a trace of inositol remained in the experimental media) and the difference between the oxygen consumption of the experimental flasks and of the control flask amounted to 0.52 to 0.57 mole of oxygen per mole of inositol.

*Preparation of scyllo-Inosose*—An excellent yield of practically pure *scyllo*-inosose can be obtained directly from 4 to 5 day inositol fermentations. The culture medium from six flasks (strain NRRL-B-474) was harvested and treated with 10 ml. of saturated lead acetate solution. The precipitate was filtered and excess lead was removed over an Amberlite IR-100 column (4 × 16 cm.). The column was washed with 200 ml. of water and the solution was concentrated to 175 ml., causing solid to separate. The addition of 175 ml. of methanol completed the crystallization. After 2 hours at 0°, the precipitate was filtered, washed with methanol, and air-dried, giving 31.4 gm. (87 per cent yield) of crude *scyllo*-inosose (decomposition 199–201°). This material may contain traces of impurity which can be removed by purification through the phenylhydrazone.

#### SUMMARY

The fermentation of inositol by *Acetobacter suboxydans* was investigated. *scyllo*-Inosose was obtained as the major product of the oxidation. No other substance was isolated in significant quantity, although various strains of *Acetobacter suboxydans* were employed and the fermentation time was varied. *scyllo*-Inosose was found to yield an osazone on prolonged treatment with phenylhydrazine. The sodium salt of the oxime of *scyllo*-inosose was prepared, and proved to be a convenient derivative for characterization purposes.

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# RADIOACTIVITY DISTRIBUTION IN THE TISSUES OF MICE BEARING MELANOSARCOMA AFTER ADMINISTRATION OF DL-TYROSINE LABELED WITH RADIOACTIVE CARBON\*

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It has long been recognized that radioactive isotopes furnish a means of irradiating deep seated neoplasms and metastases provided they can be administered in a form which will localize with a sufficient degree of selectivity in the neoplastic tissue. The availability of radiocarbon greatly expands the possibilities of this method for controlling cancer. The melanosaarcoma suggests itself for localization experiments, since heavy formation of melanin usually characterizes this tumor (1) and gives a clue to the nature of compounds which might deposit in it with the necessary selectivity. Studies are in progress in this laboratory in which various precursors of melanin labeled with  $C^{14}$  are synthesized and their metabolism followed in normal mice and in mice bearing melanosaarcoma. One such precursor, tyrosine (2), is the subject of this paper. As an exploratory experiment, DL-radio-tyrosine has been administered to mice bearing melanosaarcoma and the whole tissues have been assayed for radioactivity. The primary purpose is to assess the degree of promise which this approach offers to melanoma therapy, but the results will also have general biochemical interest. In particular, they contain information relative to the question of turnover of radioactive carbon in the body, a subject with which this laboratory is concerned in connection with a program of studying the health aspects of using radiocarbon, both in the laboratory and therapeutically.

## *Synthesis of DL-Tyrosine Labeled with $C^{14}$ in $\beta$ Position*

The method used to synthesize radiotyrosine has been described in outline elsewhere (3). The details are given below.

*p-Anisic Acid*—A solution of *p*-methoxyphenylmagnesium bromide was prepared under nitrogen from 9.3 gm. (0.050 mole) of *p*-bromoanisole and 1.32 gm. (0.055 mole) of magnesium. The reaction was started under ether, then the bulk of the bromide was added over a period of 2 hours as a solution in 40 ml. of 50 per cent ether-benzene mixture. The reaction

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mixture was refluxed during the addition, then for 6 hours longer to complete the reaction, which was sluggish. At the end of this time, 50 ml. of ether and 50 ml. of benzene were added. This was necessary to dissolve the rather insoluble Grignard reagent. The concentration of the final solution, determined by titration, was 0.276 M.

*p*-Anisic acid was prepared by carbonating 26 ml. (7.2 millimoles) of the Grignard reagent with carbon dioxide generated from 1.02 gm. (5.18 millimoles) of radioactive barium carbonate by a technique described elsewhere (4). The purified acid weighed 0.64 gm., which is 81 per cent of the theoretical yield based on barium carbonate.

*p*-Anisaldehyde—In a three-neck 30 ml. pear-shaped flask were placed 0.64 gm. (4.2 millimoles) of *p*-anisic acid and 5 ml. of purified<sup>1</sup> thionyl chloride. The two side necks were plugged, a reflux condenser was inserted in the middle neck, and the mixture was refluxed 5 hours. The thionyl chloride was then removed *in vacuo* and the residue was dissolved in 7 ml. of dry c.p. xylene. To the solution were added 0.003 ml. of quinoline-sulfur poison (5) and 0.05 gm. of 5 per cent palladium-barium sulfate catalyst;<sup>2</sup> Rosenmund reduction of the acid chloride was then carried out by passing hydrogen through the mixture at reflux temperature. The hydrogen was purified by passage through Fieser's solution (6) and drierite and was admitted to the reaction flask through a bent capillary which was inserted through one of the side necks and extended below the surface of the solution. Rising from the other side neck was a 20 cm. length of 11 mm. glass tubing, which served as an air condenser to hold back xylene. The upper 5 cm. was packed with drierite to exclude moisture. From the exit of this tube, the effluent gas was led through sodium hydroxide, in order that the hydrogen chloride evolution could be followed. The solution was violently agitated by a sealed induction stirrer<sup>3</sup> attached at the center neck. Unlubricated ground glass connections were used throughout.

The reduction required 4 hours. The yield of aldehyde in a pilot run was determined to be 73 per cent, based on anisic acid, by preparing and weighing the 2,4-dinitrophenylhydrazone. In the radioactive run the aldehyde was not isolated and its yield was not determined; the evolution of hydrogen chloride was equivalent to 88 per cent of the anisic acid.

<sup>1</sup> By distillation from quinoline, then boiled linseed oil.

<sup>2</sup> Obtained from the American Platinum Works, Newark, New Jersey.

<sup>3</sup> This stirrer was constructed with Teflon bearings and the rotor was protected with plastic paint No. 4A (Interchemical Corporation, 1073 Howard Street, San Francisco) in order to eliminate the possibility of catalyst poisons being washed into the reaction mixture by condensed xylene. Actually little or no xylene found its way past the bearing into the upper part of the stirrer.

*Anisalhydantoin*—The aldehyde solution obtained in the reduction was centrifuged to remove the catalyst, transferred to a heavy wall, 14 × 100 mm. Pyrex ignition tube, and the solvent was removed under a vacuum. To the residue were added 0.55 gm. of hydantoin, 0.7 ml. of dry diethylamine, and 1.2 ml. of dry pyridine. The tube was sealed and heated 72 hours in steam. It was opened and the solvent was pumped off at room temperature, then pumping was continued for 1.5 hours at 100°. The residue was stirred with three 2 ml. portions of hot water, each portion being removed with a filter stick before the addition of the next. The yellow residue of anisalhydantoin weighed 0.60 gm. after drying at 90°. This is a yield of 93 per cent based on anisaldehyde, assuming the yield in the reduction was 73 per cent and is in agreement with values around 90 per cent found in pilot experiments.

*Tyrosine*—To the anisalhydantoin were added 0.19 gm. of red phosphorus and 2.4 ml. of redistilled hydriodic acid, *d* 1.7. A cold finger made from a conical 5 ml. centrifuge tube was hung in the mouth of the test-tube and the mixture was refluxed 5 hours. Then 0.63 gm. of iodine was added and the mixture was refluxed 5.5 hours longer. The solution was filtered into a 30 ml. flask with a 14/20 ground neck and the solvent was pumped away. To the residue were added 5 ml. of water and the solvent was again removed. The residue was then taken up in 5 ml. of water. A yellow turbidity was present; it increased as water was added. Water was added until no further increase in turbidity was observed (25 ml.) and the turbidity was removed by centrifugation. The solution was then adjusted to pH 5.7 with ammonia, and a slight precipitate which appeared was filtered off. This appeared to be an impurity, as it turned brown on drying and was discarded. The solvent was pumped away and the residue was heated 15 minutes at 100° in a vacuum. The residue was stirred well with 5 ml. of water and the mixture was filtered. The tyrosine on the filter was washed with water and alcohol and dried at 90°. The yield was 177 mg., which is a yield of 36 per cent based on anisalhydantoin. The over-all yield, based on barium carbonate, was 19 per cent.

As a check on purity, analyses were carried out on a sample of tyrosine prepared in a pilot run by the same procedure, except that inactive barium carbonate was used for preparing the anisic acid. The analyses showed

$C_9H_{11}NO_3$ .	Calculated.	C 59.66, H 6.12, N 7.74
181.03	Found.	" 58.15, " 6.01, " 7.76
		" 58.24, " 6.15, " 7.66

The specific activity of the tyrosine was 152,300 counts per minute per mg. This is 0.9 microcurie per mg.

### *Conditions of Experiment*

Three adult male strain *dba* mice were inoculated bilaterally with melanosarcoma S91.<sup>4</sup> When the tumors were 5 weeks old, each mouse was given a single injection of tyrosine via the tail vein. The tyrosine was administered as a solution in physiological saline, prepared by dissolving 2.9 mg. of tyrosine in 0.15 ml. of 0.3 N hydrochloric acid, then adding 0.15 ml. of 0.3 N sodium hydroxide and injecting the solution promptly. Mice B and C received 0.09 ml. of this solution; Mouse A received only 0.07 ml. because the needle missed the vein on the first try.

The three mice were placed in a glass cage with provision made for collecting their excreta and for absorbing all the carbon dioxide exhaled. The mice were fed a stock diet *ad libitum*. At the end of 12 hours, Mouse A was removed from the cage and killed with nembutal; Mice B and C were killed after 24 and 72 hours, respectively.

Each mouse was dissected immediately after death into the specimens listed in Table I. The dissections were made as quantitative as possible and no tissue was discarded. This makes it possible to calculate a balance between the total amount of radioactivity administered and the amount found by assay.

### *Handling of Specimens*

The fresh tissue specimens were promptly dried in a vacuum at 60°. To see whether this drying procedure would drive volatile radioactive compounds from the tissues, the water which came off was collected and examined for radioactivity by slowly vaporizing it in a stream of oxygen and passing the gas through a combustion train. The amount of activity found in the water was less than 0.01 per cent of the administered dose and therefore this is excluded as an important source of error.

To measure the radioactivity of the specimens, they were first burned to carbon dioxide in an analytical type combustion train and the gas was absorbed in sodium hydroxide (4).

Organs smaller than 50 mg. were burned *in toto* to eliminate the effect of possible inhomogeneity in the distribution of the radioactivity in the tissue. Larger samples were ground up and an amount of tissue (100 to 150 mg.), large enough to constitute a considerable fraction of the total, was burned.

From the sodium carbonate solution obtained, barium carbonate was precipitated. When the specific activity of the barium carbonate was greater than 0.5 count per minute per mg., it was counted on an aluminum disk under a bell jar counter with a thin mica window (4). The counter

<sup>4</sup> This tumor was originally obtained from Dr. C. C. Little, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

used has an over-all efficiency of 7.7 per cent, so that 1 count per minute corresponds to 13 disintegrations per minute. All samples measured in this way were counted for a length of time sufficient to make the statistical error 10 per cent or less. Because of the fluctuations to be expected between individual animals and because of the exploratory nature of this experiment, it was not considered worth while to take the rather considerable extra time necessary to obtain count values of higher precision on the weaker samples.

Barium carbonate samples weaker than 0.5 count per minute per mg. were assayed in an ionization chamber connected to a Lindemann type electrometer.<sup>5,6</sup>

The thyroid and adrenal glands and the gallbladders were too small to furnish enough barium carbonate to work with conveniently, and in these cases benzoic acid was added to the combustion boat as carrier. The dilution introduced by this procedure is immaterial in determining the specific activity of the tissue, which is always found by dividing the total amount of radioactivity in the barium carbonate obtained by the weight of tissue burned. However, the total activity of these samples was on the lower edge of the useful range of the instrument (10 counts per minute). In addition, there is an error of 10 per cent in weighing such small organs on an analytical balance, and so there is an over-all experimental error of about 25 per cent in the values for these organs.

The adrenals and gallbladder of Mouse B and the thyroids of Mouse C deviate from the general turnover trend so strikingly that there is no doubt that these values contain gross errors in addition to those mentioned above. Each value for bone is an average from combustions in triplicate. The values obtained varied by as much as 100 per cent, presumably because of differences in amount of adhering muscle and in amount of cartilage in different bones.

*Exhaled Carbon Dioxide*—Carbon dioxide was collected by drawing through the cage a current of air which was then passed through a sintered glass disk into 1 M sodium hydroxide. The absorber was changed at intervals and the various samples of barium carbonate obtained were examined for specific activity and total activity. Since all the animals were kept in the same cage, the specific activity of the barium carbonate represents an average value over the number of mice contributing. To get the average total excretion, the total activity obtained during the first 12 hours was divided by 3, and during the next 12 hours by 2.

<sup>5</sup> This instrument was constructed by Dr. Clinton D. Janney. We are indebted to him and to Mrs. Mary Daniels for the ionization chamber measurements.

<sup>6</sup> Janney, C. D., and Moyer, B. J., A technique for the measurement of carbon 14, in preparation.

Fig. 1 shows the specific activity of the barium carbonate plotted against time. The specific activity of each sample is an average value over a time interval; to take account of this fact, at least approximately, each time value plotted as abscissa was calculated by dividing by 2 the elapsed time during an interval and adding it to total time elapsed up to the beginning of the interval.

A substantial amount of activity appeared in the exhaled carbon dioxide. The very rapid initial rise in specific activity, which reached a maximum at 1.5 hours, suggests that some of the tyrosine was extensively degraded before it had an opportunity to be incorporated into protein. During the first 12 hours, the amount of activity exhaled amounts to 19.3 per cent of the administered dose, during the second 12 hours 2.9 per cent, and during

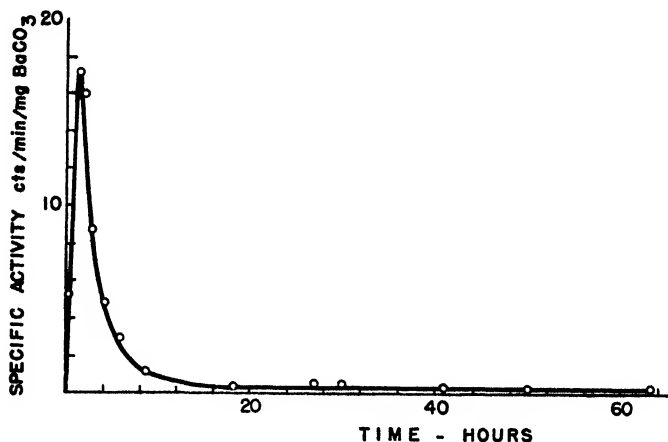


FIG. 1. Elimination of activity through the lungs as carbon dioxide

the succeeding 48 hours 7.4 per cent. Since perfusion experiments (7, 8) have shown liver to be capable of forming acetoacetic acid and carbon dioxide from tyrosine, this organ is probably responsible, in part, for the appearance of activity in the breath.

*Excreta*—The residue in the bottom of the cage, containing considerable food, was washed into a beaker and evaporated to dryness on a steam bath. From the specific activity and total weight of this material, the amount of activity eliminated in urine and feces was found to be 41.5 per cent of the administered dose. This figure does not imply that any one of the mice excreted this amount, since the three mice were all in the same cage, as explained earlier. However, if the shape of the elimination curve is similar to the shape of the carbon dioxide curve, as is likely, the individual percentages cannot be different from 41.5 per cent by an amount greater than  $\pm 25$  per cent of this figure.

In Mice A and B there was urine in the bladder at the time of dissection. The specific activities of the solid residues obtained by evaporating these samples are the ones tabulated in Table I. Since the urine production is about 3 ml. per day per mouse and the solids content is about 5 per cent,

TABLE I  
*Specific Activity (Counts per Minute per Mg. of Dry Tissue)*

Specimen	Mouse A (21.1 gm.), 12 hrs.	Mouse B (22.0 gm.), 24 hrs.	Mouse C (23.0 gm.), 72 hrs.
Adrenals.....	29	5.5*	15
Bone.....	2.62	3.03	1.96
Brain.....	7.13	3.79	4.86
Eyes.....	5.62	4.0	2.7
Gallbladder.....	28	8*	9
Heart.....	16.6	13.5	5.72
Intestines.....	31.9	21.6	10.1
Intestinal contents.....	31.9	26.2	7.41
Kidneys.....	28.6	23.9	15.3
Liver.....	20.4	14.6	11.3
Lungs.....	13.4	9.82	6.52
Lymph nodes.....	14.9	9.74	10.9
Muscle.....	5.57	3.86	2.76
Pancreas and fat pad.....	7.48	9.73	6.79
Plasma.....	44.0		
Red cells.....	1.27		
Salivary glands.....		12.8	8.28
Skin†.....	11.1	7.71	6.66
Skin and hair‡.....	4.37	4.82	4.92
Spleen.....	17.7	14.5	9.63
Stomach and contents.....	16.0	16.8	11.2
Testes.....	11.7	7.33	7.96
Thyroids.....	39	14	57*
Tumor.....	24.4	19.9	16.5
Urine.....	60.9	51.3	

\* These values are probably in error (see the text).

† The sample consisted of pieces of skin teased away from hair.

‡ The specific activity of this sample has no particular significance because of the presence of hair, which has no radioactivity except at the roots. However, the simplest way to determine the total activity in the hide was to determine the specific activity of skin plus hair and multiply by the total weight; it would be difficult to separate all the skin cleanly from hair, as would be necessary in order to obtain the skin weight.

it is clear that the urine cannot account for the total amount of activity excreted and that the feces carry a large amount.

Table I shows the specific activities of the various tissues, and Table II shows total activities and the count balance. As an approximate com-

pensation for the fact that Mouse A received only 0.07 ml. of tyrosine solution, the values obtained for this mouse, have been multiplied by 9/7 to obtain the figures entered in Tables I and II.

TABLE II  
*Total Activity (Specific Activity  $\times$  Total Weight of Specimen)*

Specimen	Mouse A, 12 hrs.	Mouse B, 24 hrs.	Mouse C, 72 hrs.
Adrenals.....	58	5.5	15
Bone.....	3,900	4,990	2,440
Brain.....	603	345	442
Eyes.....	62	44	24
Gallbladder.....	28	8	10
Heart.....	1,404	364	189
Intestines.....	11,530	8,135	4,030
Intestinal contents.....	4,940	5,240	1,530
Kidneys.....	1,990	1,990	1,690
Liver.....	6,960	5,540	4,280
Lungs.....	334	266	189
Lymph nodes.....	293	195	214
Muscle.....	8,820	4,605	2,790
Pancreas and fat pad.....	286	204	135
Plasma*	1,070		
Red cells*	578		
Salivary glands.....		424	257
Skin and hair.....	4,900	5,540	4,780
Spleen.....	422	520	222
Stomach and contents.....	631	523	549
Testes.....	311	622	358
Thyroids.....	39	9	29
Tumor.....	9,400	8,158	5,230
Urine.....	1,580	1,180	
Total.....	60,139	48,907	29,403
Total activity in carbon dioxide.....			157,900
" " " cage residue.....			90,600
			29,400
			48,900
			60,100
Total recovered, counts per min.....			387,000
" administered, counts per min.....			398,000

\* From about 0.3 ml. of blood.

Inspection of Table I shows that injected tyrosine goes to every part of the body. The ratio between the specific activity (counts per minute per mg. of dry weight) of the more active tissues (adrenals, thyroids, and intestines)

and the less active (muscle, eyes) is about 6 for Mouse A and the same for Mouse C. (Bone and erythrocytes are ignored in this comparison because of the rather special character of these tissues.) However, no correlation is found between this ratio and the ratio of turnover times; the specific activities of the corresponding tissues of Mouse C range between one-third and one-half of those for Mouse A, a difference of less than two-fold. Consequently, the specific activities cannot be regarded as a measure of the intensity of tyrosine metabolism in the various organs.

As a matter of general interest, a gross turnover time of the administered radioactivity is calculated from the values in Table II for the total amount of activity in Mice A and C at the time of death. The time for turnover of half the activity is 60 hours. This figure, which represents mainly soft tissue turnover, may be compared with the finding (9) that after administration of radiocarbonate bone retains a large part of its initial uptake for a period as long as 3 months. From the point of view of the health question, these facts suggest that in low level irradiation from ingested radiocarbon bone will be the limiting tissue which will determine the tolerable dose.<sup>7</sup>

In drawing inferences from the variations in specific activity of the various tissues, it is not unreasonable to suppose that those in which the activity is relatively high owe this to the fact that they are involved in carrying out some special function in the metabolism of tyrosine, while those which may owe their activity merely to the incorporation of tyrosine as such into the tissue proteins will be less active. The fact that all the tissues show some activity undoubtedly reflects the fact that the latter occurrence is common to all of them. On the other hand, tissues with comparatively low activity may owe it to more than simple tissue building; there is no reason why other functions must necessarily be associated with any striking concentration of radioactivity, for an individual compound may be present in high specific activity, yet if its amount is small, the gross specific activity of the tissue will not be raised appreciably. In these cases, special function will be disclosed only by identifying the chemical compounds in which radiocarbon is present. Differences in specific activity will also reflect differences in tyrosine content. In bone, for instance, a fairly low gross specific activity might be expected because of the large mineral content. In addition, the presence of blood in the tissues will contribute activity.

<sup>7</sup> The specific activity values listed for bone in Table I indicate that the half time for turnover of the activity is only a few days. However, it has been mentioned earlier that duplicate activity measurements on bone samples gave values which varied by as much as 100 per cent. Because of the magnitude of this variation, the authors do not feel that their data for bone activity are a reliable measure of turnover rate, and that the interpretation of these data should be limited to the statement that the bone uptake is comparatively low.



Despite these limitations, there are a number of tissues whose specific activities are high enough to call for some comment. In order to define them as a group, they will be arbitrarily designated as those which in Mouse A possess a specific activity greater than three times that of muscle, or 17 counts per minute per mg. The group then comprises the adrenals, thyroids, intestines, kidneys, liver, gallbladder, plasma, spleen, and tumor.

*Intestines*—The appearance of a large amount of radioactivity in the feces lends interest to the finding of high specific activity in the intestinal walls. It is known that the intestine is active in protein anabolism (10); the large fecal excretion suggests catabolism too. However, the bile represents another pathway by which DL-tyrosine metabolites could enter the feces, and the high activity of the liver and gallbladder requires that this possibility be given equal consideration.

*Adrenal Glands*—The theory that tyrosine is a precursor of epinephrine has been entertained for a long time. The formation *in vivo* of epinephrine from phenylalanine, which is known to be convertible into tyrosine, has recently been confirmed (11). It is likely that the high activity of the glands is associated with their function in synthesizing this hormone.

*Thyroid Glands*—The high specific activity of these glands is consistent with the known facts concerning thyroxine synthesis.

*Tumor*—The appearance of high specific activity in the tumor is in line with the rapidly growing character of this tissue. Whether or how much activity is due to melanin is difficult to judge. The extra activity of the tissue is not great enough to justify interpretation in terms of melanin formation. One clear conclusion is that  $\beta$ -labeled tyrosine by itself offers no promise as a therapeutic agent against melanoma.

*Plasma*—The fact that the plasma solids have a higher specific activity than most tissues is consistent with the concept (12) that plasma protein may be an intermediate source from which many tissues build their individual proteins. However, the quantitative relationships are distorted by the fact that non-protein constituents of the tissues will dilute the protein and lower the gross specific activity, while the plasma solids consist mostly of protein.

*Erythrocytes*—The low activity of the erythrocytes is interesting to consider in comparison with the finding (13) that there is no appreciable synthesis of deoxyribonucleic acid in mature erythrocytes, the low uptake of methionine (14), and the low uptake found in the tracer work of Schoenheimer and associates with nitrogen and deuterium. The activity found in our study may be entirely due to erythrocytes formed subsequent to tyrosine administration.

It is a pleasure for the authors to express their appreciation to Professor

Melvin Calvin and Professor John H. Lawrence for their interest and advice in this work.

#### SUMMARY

1. The synthesis of DL-tyrosine labeled with  $C^{14}$  in the  $\beta$  position is described.

2. The distribution of radioactivity following intravenous administration of DL-radiotyrosine to mice bearing melanosarcoma has been investigated.

3. At 72 hours, about 30 per cent of the administered dose has appeared in the breath, about 40 per cent in the urine and feces, with 30 per cent remaining in the body.

4. Radioactivity is found in every tissue of the body. The adrenals, thyroids, intestines, kidneys, liver, plasma, spleen, and tumor show the highest specific activities. Erythrocytes are lowest, with bone next.

5. In this species, the total activity in the body diminishes by half during the 60 hour period between the 12th and 72nd hour after administration of radiotyrosine.

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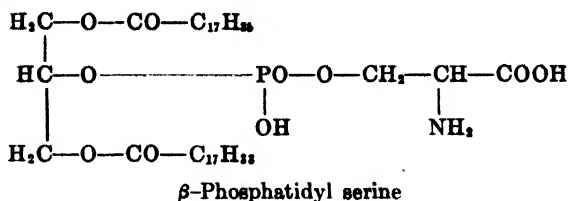
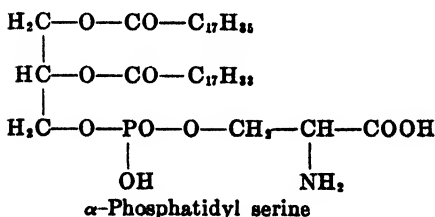
# THE CHEMICAL STRUCTURE OF PHOSPHATIDYL SERINE

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Phosphatidyl serine is the name given to a phosphatide which has been isolated from brain (1, 2). This paper reports work according to which the chemical structure of phosphatidyl serine, prepared by the method described, corresponds to one of the two accompanying formulas in which the position of the fatty acids and phosphoryl radicals is arbitrary, since there is as yet no reliable way of ascertaining whether glycerophosphoric acid in phosphatides belongs to the  $\alpha$  or the  $\beta$  form (3). The name of phosphatidyl serine was chosen because this compound appears to be an ester of serine and a phosphatidic acid (4).



The postulated formula is based on the following facts: (1) Chemical analyses of phosphatidyl serine (freed of base) for C, H, N, P, carboxyl N,  $\text{NH}_2\text{-N}$ , and fatty acids agree very closely with the theoretical values calculated from the postulated formula. (2) Glycerophosphoric acid, L-serine, and fatty acids have been isolated as cleavage products of phosphatidyl serine in molecular proportions approximately 1:1:2. (3) Phosphatidyl serine reacts with ninhydrin (5) and with  $\text{HNO}_2$  (6) in the same way as an  $\alpha$ -amino acid. This shows that both the  $-\text{COOH}$  and the  $-\text{NH}_2$  groups of serine are free in the intact molecule of phosphatidyl serine.

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On the other hand, phosphatidyl serine does not react with  $\text{HIO}_4$ . Since  $\text{HIO}_4$  is known to react with compounds with a  $-\text{CH}(\text{NH}_2)-\text{CH}(\text{OH})-$  group, and specifically with serine (7), the fact that phosphatidyl serine does not react with  $\text{HIO}_4$  is conclusive evidence that either its  $-\text{OH}$  or its  $-\text{NH}_2$  group is combined. And since its  $-\text{NH}_2$  group is shown to be free both by the ninhydrin (5) and the  $\text{HNO}_2$  reaction (6), it is obvious that the  $-\text{OH}$  group is combined. (4) From the postulated formula, phosphatidyl serine exhibits one free basic group, namely the  $-\text{NH}_2$  group, and two acidic groups, namely the  $-\text{COOH}$  group of serine and one group from phosphoric acid. This should give a strongly acidic compound that would bind 1 equivalent of base at the physiological pH. That this is the case is shown by the fact that phosphatidyl serine, isolated from brain by the use of neutral solvents and freed of water-soluble impurities by dialysis, contains K and Na, and that the ratio (equivalence of base)/(atoms of P) is 1.00. That these bases are combined with phosphatidyl serine in a salt-like combination is shown by the fact that they can be removed by treatment with 0.05 N HCl, base-free phosphatidyl serine being thereby obtained. From the free acid the original phosphatidyl serine K-Na salt can be prepared by the addition of the amount of base theoretically required.

Serine has been isolated as serine *p*-hydroxyazobenzene-*p*-sulfonate (8). It accounts for 80.7 per cent of the carboxyl N present in the starting material. From it L-serine has been recovered in a yield that accounts for 73.5 per cent of serine present in phosphatidyl serine, according to the postulated formula. The impure barium glycerophosphate obtained accounts for 81 per cent of P in starting material, and the analytically pure barium glycerophosphate for 60 per cent of the original constituent P.

Estimation of fatty acid gives for phosphatidyl serine a (moles of fatty acid)/(atoms of P) ratio of 2.00. The neutral equivalent found for the fatty acids was 283, which is the theoretical value for an equimolar mixture of oleic and stearic acids. By the lead precipitation method, phosphatidyl serine fatty acids were divided equally between saturated and unsaturated acids. From the former, pure stearic acid has been obtained. The amount obtained accounts for 69 per cent of the amount present in phosphatidyl serine, according to the postulated formula. The unsaturated fatty acid fraction appears to be mostly oleic acid, although attempts to obtain this acid in pure form have failed so far.

The iodine number calculated from the postulated formula is 31.5. The iodine number found varied between different preparations, ranging from 33.0 to 40. This discrepancy can be explained, at least in part, by the fact that our preparations were only between 92 and 97 per cent pure (as evaluated by their (carboxyl N ratio)/(total N)) and that the main

contaminant appeared to be phosphatidyl ethanolamine, which has a higher iodine number than phosphatidyl serine (2).

It must be emphasized that this paper deals only with phosphatidyl serine isolated by the method described. It accounts for about 60 per cent of all the lipide carboxyl N from brain. However, the possible existence of phosphatidyl serine having fatty acids other than stearic and oleic acids as constituents must be kept in mind. From another fraction isolated from brain cephalin, to which the name of inositol phosphatide was given for descriptive purposes (2), other preparations of phosphatidyl serine have been isolated, incidental to the isolation of diphosphoinositide (9). These phosphatidyl serine preparations are found to contain Na instead of K as the most abundant inorganic base.

#### EXPERIMENTAL

*Analytical Methods*—Manometric methods were routinely used for the estimation of C (10), P (11), N (12), and carboxyl N (5). Carboxyl N analysis on phosphatidyl serine gives better results when 1 cc. of water is added to the weighed dry material and the sample allowed to stand for a few minutes with occasional gentle shaking, so as to allow it to form an emulsion. In the case of base-free phosphatidyl serine, 1 or 2 drops of 0.1 N NaOH are added to facilitate emulsification. 13 minutes in the boiling water bath are allowed for reaction with ninhydrin, this length of time having been found to yield good checks between parallel estimations. In the later stages of work P has been estimated by Sperry's method (13), iodine numbers by Yasuda's method (14), and barium by weighing it as BaSO<sub>4</sub>. NH<sub>2</sub>-N was estimated manometrically on acid hydrolysates, as described elsewhere (2), to eliminate interference from unsaturated fatty acids (15). K was estimated as potassium phosphotungstate (16), and Na as sodium uranyl zinc acetate by a microgravimetric modification of the method of Salit (17). Ca and Mg were estimated by standard methods (18), modified to suit the type of material dealt with.

In cases in which elementary composition has been used for identification of compounds or in which values are recorded as evidence, C and H were estimated by dry combustion,<sup>1</sup> lead chromate being used in compounds that had base, N by the Dumas method, and P gravimetrically as ammonium phosphomolybdate. It has been found that C values by the wet combustion manometric method of Van Slyke and Folch (10) agreed with those obtained by dry combustion.

Glycerol was estimated by the Blix method (19), which has been found to give recoveries of only 95 to 96 per cent when tested with standard glycerol, or glycerophosphate solutions.

<sup>1</sup> Analyses run by Dr. E. W. Elek.

### *Preparation and Properties of Phosphatidyl Serine*

Phosphatidyl serine is prepared from brain cephalin by the chloroform-alcohol method of fractionation, freed of water-soluble impurities by dialysis, and lyophilized as described elsewhere (2). Usually this method yields preparations of 85 to 90 per cent purity; *i.e.*, between 85 and 90 per cent of the nitrogen present is carboxyl N. When the percentage of total N as carboxyl N is less than the stated percentage, it is easy to bring it up to this level by the following procedure.

1 gm. of the preparation under study is dissolved in 10 cc. of chloroform, and 16.5 cc. of absolute ethyl alcohol are added to the solution. A turbidity develops, and on standing or by centrifugation the system resolves itself into an underlying viscous layer and a clear supernatant solution. The clear supernatant solution is decanted, and to it are added 30 cc. of absolute ethyl alcohol. A precipitate separates which is collected and dried. On analysis it is found to contain carboxyl N at a higher concentration than the mother substance. In a typical case, by this procedure the following fractions have been obtained from 22.6 gm. of a preparation containing 1.25 per cent carboxyl N: (1) recovered from the viscous underlying layer, 7.4 gm. of material containing 1.29 per cent carboxyl N; (2) the precipitate collected from the supernatant solution, 11.7 gm. of material containing 1.43 per cent carboxyl N; (3) recovered from the supernatant solution (2), 3.2 gm. of material containing 0.35 per cent carboxyl N.

We have been unable to obtain consistently phosphatidyl serine having more than 92 per cent of its N as carboxyl N. Occasionally, preparations have been obtained showing concentrations of carboxyl N as high as 97 per cent of the total N. The main contaminant appears to be phosphatidyl ethanolamine, as is shown by the fact that all N present is  $\text{NH}_2\text{-N}$ . In search of the nature of other contaminants phosphatidyl serine was found to be essentially free of cerebrosides (carbohydrates <0.1 per cent), lecithin, or sphingomyelin (choline <0.1 per cent) and cholesterol (<0.1 per cent).

Phosphatidyl serine is obtained as a loose white powder. On being dissolved in organic solvents and recovered from solution, it acquires a light tan color. After lyophilization, phosphatidyl serine keeps fairly well if stored *in vacuo* in the dark. Under these conditions it retains a certain amount of water. For instance, 401.75 mg. of phosphatidyl serine that has reached constant weight *in vacuo* over  $\text{CaCl}_2$  at room temperature show a loss of weight of 0.65 mg. (*i.e.*, 1.65 per cent) on being heated in a vacuum at  $80^\circ$  to constant weight. Restored to former conditions (*i.e.*, in a vacuum at room temperature), it regains its former weight within 48 hours.

Phosphatidyl serine is freely soluble in chloroform, ethyl ether, and petroleum ether, and insoluble in ethyl alcohol, methyl alcohol, or acetone. On standing in contact with water, it forms an emulsion. These emulsions are very stable and an 8 per cent emulsion of phosphatidyl serine will stand centrifugation at 4000 R.P.M. for an indefinite period of time.

*Bases Combined with Phosphatidyl Serine*—Phosphatidyl serine preparations obtained by the method described are found to contain 1 equivalent of inorganic base for each atom of P. K is by far the most abundant base with a smaller amount of Na, but neither Ca nor Mg is present. Detailed results on this point are given in Table I for the two preparations. Studies carried out on cephalin preparations (20) (presumably containing about

TABLE I  
*Analysis of Two Preparations of Brain Phosphatidyl Serine*

Components	Preparation Oct. III	Calculated for $BC_{44}H_{77}O_{10}NP^*$	Preparation Sept. IIbII	Calculated for $BC_{44}H_{77}O_{10}NP^\dagger$
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
C	60.8	61.1	61.71	61.50
H	9.32	9.50	9.37	9.60
N	1.65	1.70	1.65	1.71
P	3.75	3.76	3.76	3.78
COOH-N	1.52	1.70	1.57	1.71
Iodine No.	40		38.0	

\* B stands for the cations present. Preparation Oct. III contained 3.76 per cent K and 0.51 per cent Na; (equivalence of base)/(atoms of P) = 0.98. The values given in this column have been calculated for oleylstearyl-glycerolphosphoryl serine corrected for the cations present.

† B stands for the cations present. Preparation Sept. IIbII contained 2.93 per cent of K and 0.99 per cent of Na; (equivalence of base)/(atoms of P) = 0.98. The values given in this column have been calculated for oleylstearyl-glycerolphosphoryl serine corrected for the cations present.

50 per cent phosphatidyl serine (21)) have shown that these bases are combined in un-ionized form.

*Analyses of Phosphatidyl Serine*—The results of chemical analysis of phosphatidyl serine are given in Table I. It is seen that the results obtained closely agree with values calculated for the postulated formula corrected in each case for the amount of inorganic bases present in the preparation.

#### *Preparation of Base-Free Phosphatidyl Serine*

The ability of phosphatidyl serine to form fairly concentrated emulsions in water depends upon the presence of K or Na combined in its molecule.



Acidification of the emulsion results in progressive precipitation of phosphatidyl serine as free acid. This precipitation is complete at about pH 1.5 (0.05 N HCl).

The insolubility of the free acid in water is made use of to prepare base-free phosphatidyl serine. The method is as follows.

3 gm. of phosphatidyl serine are emulsified with 150 cc. of water and to the emulsion are added 15 cc. of N HCl. The precipitate that forms is centrifuged off and washed once with 0.1 N HCl. The washed precipitate is transferred to a cellophane sausage casing and dialyzed against distilled water in the ice box for 3 days, the outside liquid being renewed six times. In the course of dialysis some of the precipitate goes back into emulsion. From the contents of the dialysis sack base-free phosphatidyl serine is obtained either by lyophilization or by precipitation with a large excess of alcohol (four to six times as much alcohol as water). In either case the dry substance is taken up in 10 cc. of  $\text{CHCl}_3$  and 70 cc. of acetone are added

TABLE II  
*Analysis of Base-Free Phosphatidyl Serine*

Component	Preparation Oct. III	Preparation Nov. III	Preparation Sept. IIIbII	Preparation Jan. III	Calculated for $\text{C}_{42}\text{H}_{80}\text{O}_{10}\text{NP}$
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
C	63.34	63.89	63.53	63.5	63.9
H	9.92	9.72	9.87	9.82	10.1
P	3.89	3.83	3.96	3.86	3.92
N	1.74	1.80	1.81	1.69	1.78
Base	1.0	0.5	0.1	0.4	0.0
COOH-N	1.59	1.65	1.68	1.59	1.78

to it. The precipitate that forms is collected and dried, 2.3 gm. of a light tan loose powder being obtained. After being dried to constant weight over  $\text{CaCl}_2$  in a vacuum, it still contains about 1.80 per cent water. For instance, 255.54 mg. of base-free phosphatidyl serine that had reached constant weight *in vacuo* over  $\text{CaCl}_2$  showed a loss of weight of 4.64 mg. (*i.e.*, 1.80 per cent) on being heated in a vacuum at  $80^\circ$  to constant weight. The dried product, when placed in a vacuum desiccator over  $\text{CaCl}_2$ , regained its former weight in 24 hours.

The results of chemical analyses of several preparations are given in Table II. They agree closely with values calculated for oleylstearyl-glycerolphosphoryl serine ( $\text{C}_{42}\text{H}_{80}\text{O}_{10}\text{NP}$ ).

After drying, base-free phosphatidyl serine forms emulsions in water with great difficulty. It is only on addition of the amount of bases (KOH, NaOH) required to reform the K or Na salt (1 equivalent of base per atom of P) that an aqueous emulsion is easily obtained.

### *Effect of Storage on Phosphatidyl Serine*

On storage *in vacuo* in the dark at room temperature phosphatidyl serine does not change its elementary composition, but some sort of rearrangement takes place in the molecule, so that the concentration of carboxyl N drops sharply.  $\text{NH}_2\text{-N}$  decreases to a much lesser degree. The results of analyses on one preparation (Nov. III) stored under the conditions given above were as follows:  $\text{COOH-N}$ : original 1.62 per cent; after 3 months 1.39 per cent; after 5 months 1.32 per cent; after 10 months 1.25 per cent; after 19 months 1.20 per cent; after 26 months 1.14 per cent. On the other hand,  $\text{NH}_2\text{-N}$ , which was 1.70 per cent originally, was 1.37 per cent after 26 months. These changes with storage are much less marked in the case of base-free preparations. For instance, base-free Preparation Nov. III, containing originally 1.65 per cent  $\text{COOH-N}$  and 1.80 per cent  $\text{NH}_2\text{-N}$  was found to contain 1.50 per cent  $\text{COOH-N}$  and 1.70 per cent  $\text{NH}_2\text{-N}$  after 26 months storage. The fact that the  $\text{NH}_2\text{-N}$  values decrease to a much lesser degree than carboxyl N values suggests that the  $-\text{COOH}$  group is mainly involved in the observed change.

Storage in a  $\text{CHCl}_3$  solution at  $-72^\circ$  (dry ice box) appears to be more satisfactory. Preparations stored this way for 18 months have shown no change in composition.

### *Isolation of L-Serine from Phosphatidyl Serine*

2.25 gm. of phosphatidyl serine containing 1.42 per cent carboxyl N were freed of base by emulsification in water and precipitation of the phosphatide by addition of HCl up to 0.1 N concentration. The precipitate was washed once with 0.1 N HCl and next hydrolyzed by boiling 6 N HCl under reflux for 3 hours. After cooling, the hydrolysate was freed of fatty acids by filtration, and the filtrate was concentrated to 4 cc. volume. To it were added 800 mg. of *p*-hydroxyazobenzene-*p*-sulfonic acid, which was dissolved by heating in the boiling water bath. Crystals formed on standing overnight in the ice box were centrifuged in the cold and washed twice with 3 cc. of ice-cold water. The washed crystals were recrystallized twice from 4 cc. of water.

After drying at  $100^\circ$  the crystals weighed 710 mg. On analysis they proved to be serine *p*-hydroxyazobenzene-*p*-sulfonate. They accounted for 80.7 per cent of the carboxyl N present in the starting material. The results were as follows:

$\text{C}_{18}\text{H}_{17}\text{O}_7\text{N}_2\text{S}$ .	Calculated.	C 47.0,	H 4.44,	carboxyl N 3.66,	base 0.00
	Found.	" 46.7,	" 4.39,	" 3.62,	" 0.3

569 mg. of this compound were dissolved in water, lead acetate was added to the solution, the lead salt removed by filtration, and the filtrate freed of traces of *p*-hydroxyazobenzene-*p*-sulfonic acid with charcoal.

The filtrate from the charcoal was dried, the residue dissolved in 2 cc. of water, and 20 cc. of alcohol were added to the solution. The crystals formed on standing overnight in the ice box were collected and dissolved in 0.5 cc. of water, and 10 cc. of alcohol were added to the solution. After standing overnight in the ice box, the crystals that formed were collected, dried, and analyzed.

The crystals weighed 142 mg. On analysis they proved to be L-serine, and accounted for 91 per cent of the serine present in the serine *p*-hydroxy-azobenzene-*p*-sulfonate or 73.6 per cent of the carboxyl N present in the starting phosphatidyl serine. The results were as follows:

$C_2H_7O_2N$ . Calculated.	C 34.2, H 6.67, N 13.32, carboxyl N 13.32
Found (corrected for 0.95% base).	" 34.15, " 6.55, " 13.22, " " 13.22

*Rotation*—A solution in 1 N HCl containing 47 mg. of crystals per cc showed in a 1 dm. tube a rotation of  $+0.67^\circ$  with sodium light;  $\alpha_D^{20} = 14.3^\circ$ . Fischer and Jacobs give  $+14.5^\circ$  (22).

#### *Isolation of Glycerophosphoric Acid from Phosphatidyl Serine*

3.35 gm. of phosphatidyl serine (Preparation May III-41) were emulsified in 150 cc. of  $H_2O$ , and 15 cc. of N HCl were added to the emulsion. The resulting precipitate was centrifuged, washed once with 0.1 N HCl, and then hydrolyzed with 6 N HCl for 100 minutes in the boiling water bath. After cooling, the hydrolysate was filtered and the filtrate evaporated to dryness in a vacuum. The dry residue was taken up in 50 cc. of water and the solution treated with 1 gm. of  $Ag_2O$  after the addition of 0.5 cc. of concentrated acetic acid. The precipitate was filtered off and the silver was removed with hydrogen sulfide. The silver sulfide was filtered off, after which the filtrate was evaporated to dryness. The residue was dissolved in 30 cc. of  $H_2O$  and brought to pH 10 by the addition of 12 cc. of saturated  $Ba(OH)_2$ . The precipitate that separated was centrifuged off and washed twice with water. To the clear solution an equal volume of alcohol was added and the precipitate that separated was centrifuged off and washed once with 50 per cent alcohol and dried in a vacuum at  $142^\circ$  to constant weight. It weighed 831.2 mg. On analysis it was found to contain 11.1 per cent P, 32.5 per cent glycerol, 32.76 per cent Ba, and 0.2 per cent N. It accounted for 81 per cent of the P present in the starting material. It appeared to be barium glycerophosphate mixed with acid barium glycerophosphate.

Four precipitations from water solution, by addition of an equal amount of alcohol, brought the nitrogen concentration down to 0.03 per cent. Finally, the Ba salt was dissolved in 50 cc. of  $H_2O$ , the solution was made

strongly alkaline by addition of 10 cc. of saturated aqueous  $\text{Ba}(\text{OH})_2$  solution, and an equal volume of alcohol added to it. The alcohol-insoluble precipitate was centrifuged, washed twice with cold 50 per cent alcohol, dried, redissolved in 30 cc. of water, and  $\text{CO}_2$  bubbled through the solution. A slight precipitate was removed by centrifugation and to the supernatant was added an equal volume of methyl alcohol. The precipitate that formed was centrifuged, washed with 50 per cent methyl alcohol, and dried to constant weight at  $142^\circ$ .

575 mg. of material were thus obtained. On analysis it proved to be Ba glycerophosphate. The analytical results were as follows:

$\text{C}_2\text{H}_7\text{O}_5\text{P Ba}$ .	Calculated.	C 11.73, P 10. 1, Ba 44.5, glycerol 29.3
	Found.	" 11.69, " 10.05, " 44.6, " 28.7

#### *Estimation of Fatty Acids*

Phosphatidyl serine was saponified with 8 per cent alcoholic  $\text{NaOH}$  by refluxing for 4 hours. After cooling, the solution was nearly neutralized with hydrochloric acid and concentrated to dryness *in vacuo*. The residue was acidified with  $\text{N HCl}$  and the fatty acids were extracted with four successive portions of ether. The combined ether extracts were washed three times with equal volumes of water, and the washed ethereal extract was evaporated to dryness. By this method, saponification appeared to be complete in 4 hours, the same results being obtained for periods of saponification of 4 and 8 hours.

A number of preparations were thus analyzed. The purest ones (containing 97 per cent of total N as  $\text{COOH-N}$ ) yielded 68.6 per cent of the weight of the starting material as fatty acids, neutral equivalent 283; (moles of fatty acid)/(atoms of P) = 2.00. The theory for the K salt of phosphatidyl serine assumed to contain oleic and stearic acid radicals is 68.8 per cent of the starting material as fatty acids and the neutral equivalent for an equimolar mixture of oleic and stearic acids is 283. Preparations of lesser purity yielded fatty acids of higher neutral equivalent (up to 291).

*Isolation of Component Fatty Acids*—4 gm. of phosphatidyl serine were emulsified in 300 cc. of  $\text{H}_2\text{O}$ , and 25 cc. of 19.2  $\text{N NaOH}$  were added to the emulsion. The solution was immersed in a boiling water bath for 6 hours. After cooling, the solution was acidified with  $\text{HCl}$  to pH 1.5 and allowed to stand overnight in the ice box. The next morning it was extracted four times in succession with an equal volume of ether each time. The ethereal extracts were combined and washed twice with an equal volume of water, after which the solution was evaporated to dryness. The residue weighed 2.7 gm.

The fatty acids were separated into saturated and unsaturated acids by the method of Twitchell (23) as follows: The residue was dissolved in 80 cc.

of alcohol in a 100 cc. centrifuge tube immersed in a water bath at 75°, and to the hot solution were added in succession 0.5 cc. of acetic acid and 7 cc. of a 25 per cent aqueous neutral lead acetate solution. A small amount of precipitate settled to the bottom. The clear supernatant solution was transferred to another centrifuge tube in the same bath and then the bath was allowed to cool. Next morning the clear supernatant was decanted and the precipitate was washed with alcohol. The combined washing and supernatant solutions were evaporated to dryness. The residue was dissolved in 30 cc. of ether and the ethereal solution allowed to stand overnight in the ice box. The small amount of precipitate formed was filtered off and discarded. The filtrate was evaporated to dryness and the residue dissolved in 40 cc. of methyl alcohol, after which the lead was removed with hydrogen sulfide. The filtrate from the lead sulfide was evaporated to dryness. The residue was an oil which contained some white crystals. It weighed 1250 mg.

The oily residue was treated with 30 cc. of petroleum ether. Most of it went into solution. After standing overnight in the ice box, the petroleum ether solution was filtered and the filtrate evaporated to dryness. The residue was a clear oily liquid and weighed 1100 mg. The neutral equivalent was 283 and the iodine number 87.0. It appeared to be slightly impure oleic acid. Attempts to isolate analytically pure oleic acid from it have failed. The amount of impure oleic acid obtained amounted to 79.5 per cent of the amount present in the starting material, according to the postulated formula.

The alcohol-insoluble lead soaps were reprecipitated from 60 cc. of hot alcohol (to which 0.5 cc. of acetic acid had been added), the solution being allowed to cool over a period of 2 hours. After standing overnight at room temperature, the precipitate formed was collected by centrifugation and washed with 95 per cent alcohol.

After drying in a vacuum, the lead soaps were transferred to a separatory funnel with ether and the ethereal suspension washed with dilute nitric acid and then four times with water, after which the ethereal solution was evaporated to dryness. The residue was a crystalline mass which weighed 1100 mg., m.p. 65°. Neutral equivalent 279; iodine value <1.0. It was assumed that the material was impure stearic acid. The residue was dissolved in 80 cc. of hot alcohol. On cooling, a small amount of precipitate separated which was filtered off and the filtrate evaporated to dryness, after which the residue was dissolved in 40 cc. of hot alcohol. On cooling, a small precipitate formed. The solution was filtered, evaporated to dryness, and the residue dissolved in hot alcohol. No precipitate formed on cooling. The solution was evaporated to dryness.

The crystalline residue weighed 950 mg. On analysis it proved to be pure stearic acid. The results were as follows:

$C_{18}H_{34}O_2$ .	Calculated.	C 76.0, H 12.72, Base
	Found.	" 75.8, " 12.65, " 0.1

M.p. 69.3°; mixed m.p. with stearic acid, 69.3°; neutral equivalent 284.0.

The amount of pure stearic acid obtained represents 69 per cent of the amount present in the starting material, according to the postulated formula.

#### SUMMARY

1. A method is described for the isolation of phosphatidyl serine of at least 92 per cent purity.

2. As cleavage products, glycerophosphoric acid, L-serine, and fatty acids have been isolated in molecular proportions of 1:1:2. Fatty acids present appear to be mainly stearic acid and oleic acid.

3. Phosphatidyl serine reacts with  $HNO_2$  and ninhydrin as an  $\alpha$ -amino acid, which shows that both the  $-COOH$  and the  $-NH_2$  groups are free. It does not react with  $HIO_4$ , which shows that either its  $-NH_2$  or its  $-OH$  group is combined. Therefore, it appears that the combination of serine in the phosphatidyl serine molecule is through its  $-OH$  group.

4. It is concluded that the structure of phosphatidyl serine is that of stearyloleylglycerolphosphoryl serine. The results of analyses are found to agree with the values calculated from the postulated formula.

5. As isolated by neutral solvents, phosphatidyl serine is obtained as a K and Na (the former being the most abundant) salt, (equivalence of base)/(atoms of P) = 1.00. The inorganic cations can be removed by treatment with 0.1 N HCl.

6. Phosphatidyl serine studied in this paper represents 60 per cent of total lipide carboxyl N in brain. Other preparations of phosphatidyl serine can be obtained in which Na is the most abundant base.

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## BACTERIMETRIC STUDIES

### I. FACTORS AFFECTING THE PRECISION OF BACTERIAL GROWTH RESPONSES AND THEIR MEASUREMENT\*

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The accuracy of bacteriological amino acid determinations is generally considered to be of the order of magnitude of 10 per cent and attainment of optimal results often depends upon the statistical effect of large numbers of individual experiments. By contrast, the order of magnitude of the accuracy of specific chemical methods of analysis is greater by a power of 10 and adequate results are obtained from duplicate or triplicate determinations. As long as few data are available concerning the rôle of the many variables which affect microbiological analysis, the presumption that biological factors represent the dominating sources of error remains unverified. The present paper records results of a study of various factors which affect precision and reproducibility of quantitative measurements of growth responses of *Streptococcus faecalis* (American Type Culture Collection No. 9790).

*Medium*.—Initially, the medium and method were those of Stokes *et al.* (2). Subsequently photoelectric turbidimetry was adopted as the method of measurement because, among other reasons, it removes the limitations on the buffering capacity of the medium imposed by the acidimetric method. The endeavor to increase the range of linear response to increments of the limiting nutrient by minimizing changes of pH during growth and considerations regarding completeness<sup>1</sup> of the medium in terms of growth fac-

\* Aided by a grant from Mrs. L. Elizabeth Nax. Reported in part before the American Society of Biological Chemists, Chicago, May, 1947 (1).

<sup>1</sup> For present purposes a medium is termed *complete* with regard to a growth factor if its effective level is not changed by the addition of the material to be analyzed. When a medium is ready for sterilization and inoculation after its component volumes have been combined in the individual culture tubes, it is a *finished* medium. If it is prepared in bulk and placed into culture tubes in a single operation, it may be called a *prepared* medium. Media which, for analytical purposes or other reasons, contain certain components in submaximal amounts will be designated as *limited* media, and accordingly a specific medium which contains all of its components in maximal amounts is called a *non-limited* medium. The term *basal* medium adequately identifies the form in which a medium (usually limited) is made in bulk; *i.e.*, usually at twice the concentration of the finished medium.



tors led to the adoption of the provisional modified medium<sup>2</sup> shown in Table I. This medium differs from that of Stokes *et al.* (2) as follows: The glucose content is increased from 1.00 to 2.00 per cent; the 0.0066 M potassium phosphate buffer of pH 6.7 is replaced by 0.3 M sodium phosphate and, to satisfy potassium requirements (3), 0.005 M potassium phosphate buffer of pH 6.5; adenine, guanine, and uracil are replaced by a hydrolysate of

TABLE I  
*Composition of Modified Buffered Medium*

Constituent	Amounts per culture tube (10 cc.)	
		mg.
Glucose		200
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	1.95	269.4
Na <sub>2</sub> HPO <sub>4</sub>	1.05	149.1
Sodium acetate (anhydrous)		60.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>		6.00
DL-Glutamic acid		6.00
KH <sub>2</sub> PO <sub>4</sub>	0.0325	4.42
K <sub>2</sub> HPO <sub>4</sub>	0.0175	3.05
Yeast nucleic acid + 10% thymine, hydrolyzed*		2.20
MgSO <sub>4</sub> ·7H <sub>2</sub> O		2.00
Glycine		2.00
Alanine, aspartic acid, isoleucine, leucine, methionine, phenylalanine, serine, threonine, valine (all DL)		2.00 (each)
Arginine, cystine, histidine, hydroxyproline, proline, tryptophan, tyrosine (all L)		2.00 "
L-Lysine		1.00
NaCl, FeSO <sub>4</sub> ·7H <sub>2</sub> O, MnSO <sub>4</sub> ·4H <sub>2</sub> O		0.100 (each)
L-Asparagine, L-glutamine		0.050 "
Nicotinamide		0.010
Pyridoxamine		0.0040
Pantothenic acid, riboflavin, thiamine hydrochloride		0.0020 (each)
p-Aminobenzoic acid		0.00040
Biotin		0.000050
Pteroylglutamic acid		0.000020

\* 500 mg. of yeast nucleic acid (Schwarz Laboratories) and 50 mg. of thymine are digested with 5 cc. of 3 N HCl for 6 hours at 120° in a sealed tube. The dark filtrate is made up to 50 cc. 0.2 cc. of this solution is used for each 10 cc. of finished medium.

yeast nucleic acid and thymine (as a substitute for not readily available thymonucleic acid); norleucine is omitted because it is apparently not a protein component (4), but may, on the other hand, act as a metabolic competitor of methionine (5); glutamic acid is increased from 2 to 6 mg.

<sup>2</sup> Characterization of this medium in terms of analytical results will be the subject of another paper.

because it appears to have a favorable effect on generation time (6); biotin is increased twenty-five times because highly diluted solutions were found to be extremely unstable and because 0.002  $\gamma$  seemed to be less than twice the amount required for maximal growth under our conditions; ammonium sulfate, asparagine and glutamine, and the change from nicotinic acid to nicotinamide are introduced as potential improvements in the light of recent developments (7).

*Sterilization*—An experimental evaluation of the average precision of a single determination (titration of a single tube), when made according to Stokes *et al.* (2), showed a standard deviation of 5 to 6 per cent, so that seven

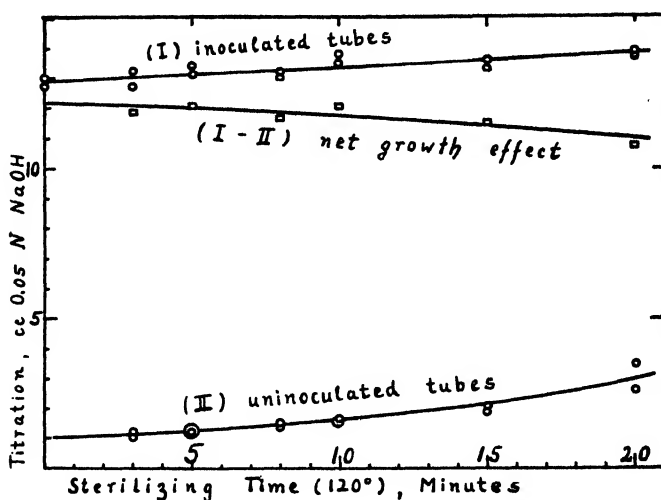


FIG. 1. Sterilizing time and acid formation. Seven sets of four tubes, containing 10 cc. of a prepared non-limited Stokes' medium, were separately sterilized at 15 pounds steam pressure for periods ranging from 3 to 20 minutes. After inoculation of two tubes of each set, all were incubated (37–38°) and titrated.

to ten determinations would be required to yield, for a single point, an average value with a standard error of  $\pm 2$  per cent.

Fig. 1 summarizes the results of an experiment which shows that bacterial growth, as reflected in net acid production, is adversely affected by extended heating of the medium, thus suggesting that local variations in the intensity of heat sterilization may be causes of low reproducibility. Similar observations have been reported by Hill and Patton (8). In the procedure finally adopted, as a result of these and other findings, the steam pressure of the sterilizer is kept at 15 to 17 pounds for not more than 2½ minutes. This technique has proved to be completely adequate; in many dozens of experiments, no growth has been observed in uninoculated control tubes.

The experimental results shown in Table II<sup>3</sup> indicate that individual variations in sterilizer action are not likely to give rise to more than minor differences in subsequent growth when the 2½ minute procedure is employed.

*Photoelectric Measurement*—Three aspects of the practicability of turbidimetric measurement are the availability of a suitable instrument, culture tubes of uniform bore, and the possible interference of color. As will be shown in detail, the photoelectric Coleman model 11 spectrophotometer possesses adequate accuracy and sensitivity, and the Coleman cuvettes

TABLE II

*Effect of Individual Sterilizations on Subsequent Color Formation and Bacterial Growth*

		Group A*	Group B*	Group C*
Sterilization	Time required to reach 15 lbs. pressure, sec.	40†	25	25
	Total time at or above 15 lbs. pressure, sec.	165	170	170
	Highest pressure reached, lbs. per sq. in.	17½	18	18½
	Cooling time (return of pressure to 0), min.	10	10	10
	Optical density, mean values of 4 tubes, with standard error			
Incubation (water bath, 37.7°)	After 22 hrs., inoculated	181 ± 1	181 ± 1	182 ± 1
	“ 22 “ uninoculated	36 ± 2	38 ± 2	35 ± 1
	After 44 hrs., inoculated	320 ± 2	322 ± 2	319 ± 1
	“ 44 “ uninoculated	100 ± 4	106 ± 1	105 ± 5
	Net value of bacterial turbidity 22 hrs.	145 ± 2	143 ± 2	147 ± 1
	44 “	220 ± 4	216 ± 2	214 ± 5

\* All tubes contained 10 cc. of prepared modified medium limited by 12 γ of threonine. Each group consisted of eight tubes, four of which were inoculated.

† The sterilizer was cold in the beginning.

(19 × 150 mm. round, selected) are of sufficient uniformity to permit their use as culture tubes which can be directly read in the instrument.

Fig. 2 shows absorption spectra of a culture medium in the presence and absence of a protein hydrolysate. Condensed results of an experiment involving measurements at various wave-lengths and concerned with the effect of sterilization time and colors upon bacterial growth in turbidimetric terms are shown in Table III. Tubes containing a prepared non-limited

\* All spectrophotometric values are reported in optical density units,  $1000 \times \log I_0/I$  ( $I_0$  = intensity of incident light,  $I$  = intensity of light after passage through the medium measured).

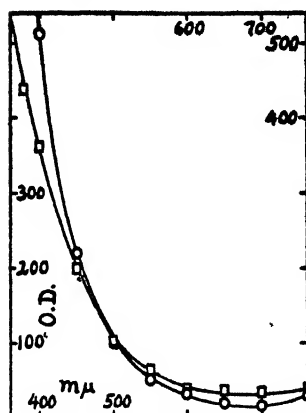


FIG. 2. Absorption spectrum of Stokes' medium. Optical density readings of a standard culture tube (10 cc. of finished medium). ○, 5 cc. of basal medium (non-limited), after 20 minutes sterilization; □, 5 cc. of the same medium and the neutralized hydrolysate of 2.9 mg. of tissue protein (selected because among six hydrolysates it had the deepest color); 5 minutes sterilization and 16 hours incubation.

TABLE III

*Gross and Net Optical Density Values of Bacterial Turbidities under Different Conditions*

	Wave-length	Sterilization			
		10 min.		20 min.	
		Incubation, 37-38°			
		2 days	4 days	2 days	4 days
	mμ				
Total reading.....	400	1380	1580	1550	1740
Net reading, I*.....	400	810	870	660	700
“ “ II†.....	400	910	1030	740	830
Total reading.....	500	1010	1170	1060	1230
Net reading, I.....	500	850	970	800	900
“ “ II.....	500	890	1030	850	1000
Total reading.....	700	730	830	690	790
Net reading, I.....	700	690	790	630	720
“ “ II.....	700	690	790	650	750

\* Obtained by deducting color of non-inoculated tubes.

† Obtained by deducting color of inoculated tubes after centrifugation.

Stokes medium were sterilized at 15 pounds pressure for 10 and 20 minutes, respectively, and all except two tubes of each set were inoculated. The formation of color during both sterilization and incubation is evident, as is

the fact that the bacteria adsorb or consume some of the color of the medium; after centrifugation non-inoculated tubes show more color than inoculated ones. However, at 700  $m\mu$  and with 10 minutes (or less) of sterilization, this difference becomes negligible. Therefore, readings were subsequently made at 700  $m\mu$  and color correction values were obtained from control tubes without bacterial growth. A tube containing water was used for zero adjustment and cultures were shaken before measurement to insure uniform suspension.

*Mechanical Factors Affecting Precision of Photometric Readings*—An evaluation of the following five factors was undertaken: (a) reproducibility of individual spectrophotometric readings of transparent media, (b) uniformity of the transmittancy of the glass of the tubes, (c) uniformity of the

TABLE IV  
*Statistical Analysis of Mechanical Factors in Photometric Readings*

Series No.	No. of tubes	Medium in optical path	Variate measured	Mean reading	Standard deviation
1	88	H <sub>2</sub> O	Difference between 2nd and 1st determinations for each tube	0	$\pm 1.8$
2	21	CuSO <sub>4</sub>	" "	488	$\pm 1.3$
3	69	H <sub>2</sub> O	Difference between readings of individual tube and mean value of all tubes	0	$\pm 1.5$
4	59	CuSO <sub>4</sub>	" "	487	$\pm 2.3$
5	59	Bacterial suspension	" "	486	$\pm 2.9$
6	59	CuSO <sub>4</sub> or bacterial suspension	Difference between readings of individual tube with CuSO <sub>4</sub> solution and bacterial suspension	487	$\pm 1.8$

diameter of tubes as determined with a transparent medium or (d) with a bacterial suspension, and (e) reproducibility of readings of a bacterial suspension. The results of the measurements are summarized in Table IV. In these tests the tubes were filled with water, a copper sulfate solution, or a suspension of heat-killed bacteria.

In the statistical analysis of the results the approximation formulas given by Daniels (9), involving the use of the term,  $K = \pm 0.845/(n\sqrt{n-1})$ , have been used. The standard error,  $M$ , is then equal to  $\pm 1.48K_n\sum v$ , and the standard deviation,  $m$ , is equal to  $\pm 1.48\sqrt{n}K_n\sum v$ , where  $n$  is the number of measurements and  $\sum v$  the sum of the deviations from the mean.

Within each series, the distribution of individual values was found to approximate closely that to be expected from a normal distribution (in the

statistical sense (10)); *i.e.*, one-third of the values falls beyond the range of  $\pm 1m$ , and one-twentieth exceeds the range of  $\pm 2m$ . The data of Series 1 and 2 indicate that in terms of optical density units the error due to instrument, human factor, and individual tube corresponds to a standard deviation of less than  $\pm 2$ . According to Series 3, variations in the transmittancy of the glass of the individual tubes do not exceed the basic reading error. The results of Series 4 and 5 show that variations in the diameter of tubes contribute a small but definite additional error. According to Series 5, measurements of bacterial suspensions appear somewhat less precise than those of transparent media. This is confirmed in Series 6, which also shows that tube calibration values obtained with copper sulfate are valid for bacterial readings.

If one uses a supply of tubes showing normal distribution in size (optical path) with a standard deviation of approximately  $\pm 0.5$  per cent, variations of tube size will not, in the average, introduce an error appreciably larger than that inherent in the spectrophotometric reading itself.

*Pipetting and Evaporation*—In actual analytical work, a composite volume of, usually, 10 cc. is measured into each tube by several pipettings. Sixteen 2 cc. portions of water, delivered from different parts of the scale of serological pipettes of 5 cc. capacity, averaged, according to their weights, 2.004 cc., with a standard deviation of  $\pm 0.015$  cc.

Twelve tubes containing growing 10 cc. cultures kept for 24 hours in the water bath at  $42^\circ$  (*i.e.* several degrees above the usual incubation temperature) lost by evaporation  $2.13 \pm 0.34$  (s.d.) per cent of their weight. It is apparent that these two sources of error remain in magnitude below that of tube dimensions.

*Cleaning of Tubes*—In the course of actual assay studies instances were encountered periodically of single tubes being far behind their expected turbidity values when readings were taken after 20 hours of incubation or earlier. In most cases the anomaly had disappeared when additional readings were taken 24 or more hours later. As a result of these observations a study was made of cleaning methods. Washing with a dilute solution of Nacconol (a long chain alkyl aryl sodium sulfonate detergent) followed by numerous rinsings had been the initial practice. The following procedure, adopted as a result of many experiments, has consistently proved to yield a dependable supply of clean tubes. The used tubes are washed with tap water, drained, and submerged in cleaning fluid<sup>4</sup> and kept on the steam bath

<sup>4</sup> A simple method of preparing this solution is as follows: Make a hot solution of equal weights of water and sodium dichromate. To this solution, placed in a large porcelain pot, add, with stirring (and great care), approximately 11 times its weight of concentrated sulfuric acid. This solution is used repeatedly until the green color of reduced chromium becomes noticeable.

overnight. After removal of the cleaning fluid by thorough rinsings with tap water and at least one rinsing with distilled water, the tubes are submerged in distilled water in covered beakers and autoclaved at 15 pounds pressure for 20 minutes.

*Incubation Temperature; Air Incubator versus Water Bath*—The temperature dependence of the growth rate of the organism used has been made the object of a separate investigation, begun after completion of the experiments here described. In accordance with prevailing practice an incubation temperature of approximately 37–38° has been used throughout the present work. However, when it appeared that, in the incubator used (a Hearson anhydric electric of 20 × 20 × 24 inches inside dimensions), reasonable temperature uniformity, as measured in terms of bacterial growth, exists only within a narrow space, a comparison was made of three different facilities: System A, the Hearson incubator, with the shelf placed at medium height, occupied by six wire racks, each of an area of 10½ × 4½ inches, providing a total of 240 test-tube spaces; System B, a modern incubator with forced air circulation provided by a twin blower system (three of the 40 tube racks were placed on the top and three similar racks on the bottom shelf); and System C, a constant temperature water bath assembled in the laboratory. This bath consists of a 36 × 13 × 16 inch aquarium tank and the requisite accessories: a high speed electrical stirrer, a 500 watt immersion heater, an adjustable mercury thermostat, a mercury plunger relay, and ledges for the support of five 40 place wire racks. For reasons of circulation all positions adjoining the walls of the tank are left vacant. During operation the bath is covered with roof-shaped pieces of sheet aluminum, and the water level is automatically maintained 4 inches above the bottom of the tubes.

The tests were run by charging a large number of tubes with prepared modified medium limited by 12 γ of threonine. The tubes were uniformly distributed throughout the racks, with empty tubes placed in the unused spaces. In the water bath (System C) all spaces were thus occupied, but in the air incubators (Systems A and B) only every second space was filled chess-board fashion in order to permit free circulation of air. All tubes were removed after approximately 22 hours and, after readings were taken, returned to be read again after a total of 44 to 48 hours of incubation. The results given in Table V show that readings are consistently more homogeneous after the second period of incubation and that the water bath gives substantially more uniform results than the air incubators. Possible errors arising as a result of additional growth taking place while the tubes are being read are eliminated by reading the series forward and backward and taking averages. Comparison of the data of Tables IV and V shows that after 2 days of incubation in the bath the degree of consistency of readings

approaches that to be expected from the operation of the non-living variables.

*Inoculum*—In the practice of microbiological assay it is customary to prepare a fresh inoculum for each day's work. Nevertheless, with identical media and identical conditions, identical amounts of growth are not generally expected in separate experiments. This lack of reproducibility may be attributed to uncontrollable day to day variations in the composition of the medium, in chemical changes incident to sterilization, in temperature and time of incubation, or in the biological activity of the bacteria. After the conditions of sterilization and incubation had been standardized, the question of the variability of inocula was examined.

TABLE V  
Results of Comparisons of Incubation Systems

System	No. of tubes	Period of incubation	Range of readings, optical density	Average value $\pm$ s.d.; optical density	No. of deviations exceeding			
					$\pm 1$ s.d.		$\pm 2$ s.d.	
					Ex-pected*	Found	Ex-pected*	Found
A. Hearson incubator	48	22	138-186	172.6 $\pm$ 11.1	16	15	2	3
			241-272	259.3 $\pm$ 7.6		13		2
B. Circulating air incubator	46	22	121-155	138.0 $\pm$ 8.5	15	12	2	0
			229-254	242.9 $\pm$ 5.2		16		2
C. Water bath	40	22	161-177	167.8 $\pm$ 4.0	13	14	2	1
			220-231	226.1 $\pm$ 3.3		14		0

\* According to "normal distribution."

We have stored and subcultured agar cultures of *Streptococcus faecalis* in the manner described by Stokes and Gunness (11). In order to study the effect of storage of the inoculum on the resulting growth the experiments described in Fig. 3 were undertaken. A slow decrease of growth potential of the inocula as a result of their cold storage is evident; the rate of this decrease, measured in terms of the resulting 16 hour growths, is approximately 3 per cent per day, and in terms of 22 hour growths, 1 per cent per day. The standard deviations within each set of eight tubes averaged  $\pm 3$  after 16 hours of incubation and  $\pm 2$  after 22 hours, and apart from slightly lower net growth no disadvantage seems to accrue from the use of stored cultures. An earlier statement concerning the advantage of the use of aged inocula (1) must be withdrawn.

In view of these observations, it became of interest to consider the effect



of the age of the parent agar culture on the growth potential of derived inoculation cultures. Accordingly, bouillon subcultures were made from five different agar cultures which had been stored at  $+4^{\circ}$  for different periods ranging from 0 to 96 days. The bouillon cultures were incubated for periods of 7 or 24 hours and, after determination of the resulting optical density, were centrifuged, washed as usual, and then diluted with water, so that suspensions of somewhat similar concentrations resulted. Four 10 cc. portions of prepared standard medium (12  $\gamma$  of threonine) were inoculated with each of these suspensions, the tubes were simultaneously incubated in the water bath, and readings were taken after 22 and 44 hours.

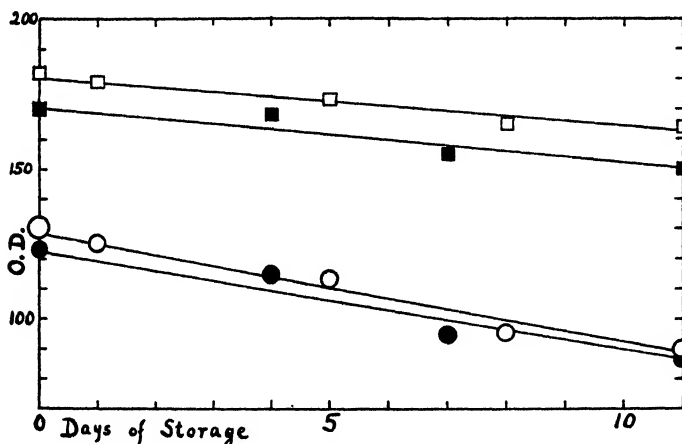


FIG. 3. Influence of storage on inoculum. Stab cultures from agar were grown in bouillon medium (8 cc.) and the resulting cultures were centrifuged, washed with saline, again centrifuged, decanted, and stored at  $+4^{\circ}$ . After dilution with 50 cc. of water, single drops were added to 10 cc. portions of prepared modified medium limited by 12  $\gamma$  of threonine and sterilized 2½ minutes. Optical density readings were taken after 16 to 17 hours (round symbols) of incubation ( $37-38^{\circ}$ ) and after 22 to 25 hours (square symbols). The open symbols show results of a series of inocula grown for 6 hours; the solid symbols refer to another series which were grown for 24 hours.

The results are summarized in Table VI. Apparently the cold storage of agar cultures for more than 3 months has no significant effect on the growth potential of their bacterial progeny, although the agar-stored bacteria themselves show a marked loss in early activity.

Because of the observed tendency for readings to become more uniform with increasing time of incubation (at least up to about 60 hours), it seemed of interest to determine to what extent the size of the inoculum may be a factor in this connection. The experiment described in Fig. 4 shows that during the terminal stages of growth, when the residual concentration of the limiting nutrient presumably becomes a rate-controlling factor, the bac-

TABLE VI  
Growth Potential of Inocula Derived from Agar Cultures of Different Age

Agar culture, duration of storage (+4°)	Derived bouillon culture			Inoculated synthetic medium (12 $\gamma$ threonine), resulting growth (mean value of 4 tubes, with standard error); optical density	
	Incubation time	Extent of growth, optical density	Water used for dilution of washed culture	After 22 hrs.	After 44 hrs.
days	hrs.		cc.		
96	24	790	100	150 $\pm$ 1	201 $\pm$ 1
89	24	770	100	149 $\pm$ 1	202 $\pm$ 1
66	24	770	100	150 $\pm$ 1	202 $\pm$ 1
66	7	200	25	148 $\pm$ 2	204 $\pm$ 2
41	24	780	100	151 $\pm$ 1	206 $\pm$ 1
0	7	720	100	152 $\pm$ 1	202 $\pm$ 2

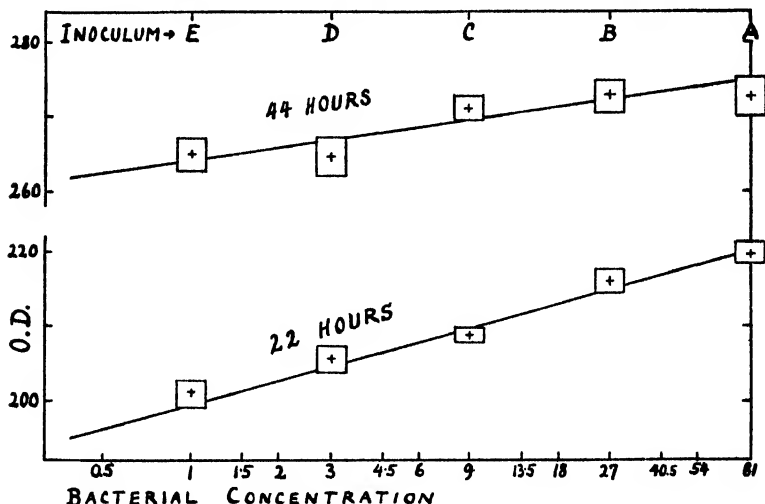


FIG. 4. Influence of size of inoculum. A freshly grown 20 hour bouillon culture was centrifuged, washed, and diluted with 10 cc. of water. Of forty-five tubes containing prepared modified medium (12  $\gamma$  of threonine; sterilized 2½ minutes) nine were inoculated with single drops of this suspension (Inoculum A). By successive dilutions with 2 volumes of water, Inocula B, C, D, and E were obtained so that the consecutive bacterial concentrations were approximately in the ratio of 81:27:9:3:1. The abscissa units are logarithms of the bacterial concentrations of the inocula; optical densities were measured after 22 and 44 hours of incubation (water bath, 37.7°). The symbols represent mean values with their standard errors.

terial density attained bears an approximately linear relationship to the logarithm of the quantity of inoculum, and that the influence of inoculum size decreases as growth approaches asymptotically the depletion level.

This finding is consistent with the conclusion of Harrington (12) that the lag period, *i.e.* the time required to reach visible turbidity, is a linear function of the bacterial count at inoculation. For the middle inoculum concentration (which corresponds approximately to the usual practice), and for the medium used, doubling or halving of the inoculum would change the optical density value at 22 hours by about 1.5 per cent and at 44 hours by about 0.6 per cent. Accordingly, ordinary variations in the size of individual inoculation drops can hardly be sources of significant variations in the analytical results.

On the basis of the foregoing the following procedure is proposed for the preparation of an inoculum of practically constant properties. A 6 hour culture in bouillon is grown from a suitable agar inoculum, the resulting optical density is measured, and, after centrifuging and washing the cells with water, the fresh inoculum is diluted with an amount of sterile water which in cc. is approximately one-tenth of the optical density; *e.g.*, for a suspension of 650 optical density 65 cc. of water are used.

For large numbers of inoculations, it is convenient to use a device made by sealing a stop-cock to one end of the bulb of a broken 25 cc. pipette and drawing out the other end to a fine tip, so that the whole piece is not longer than 9 inches. In use it is clamped at an angle about 20° above the horizontal position, and the stop-cock is adjusted to a convenient drop rate.

#### SUMMARY

The influence of experimental variables upon the growth of *Streptococcus faecalis* in synthetic media has been investigated with the following results:

1. Heat sterilization *per se* may contribute to the titratable acidity of a synthetic medium and at the same time adversely affect subsequent growth. Heating for 2½ minutes at 15 pounds steam pressure is sufficient for effective sterilization of the synthetic media investigated.

2. In photoelectric turbidimetry of bacterial cultures, errors due to color effects are smallest at wave-lengths near 700 mμ. Color differences which exist between non-inoculated and inoculated but otherwise identical media after incubation become negligible when the sterilization time is shortened and readings are taken at 700 mμ.

3. A distribution pattern among the optical diameters of selected culture tubes corresponding to a standard deviation of ±0.5 per cent adequately matches the precision of turbidity measurements obtained with the Coleman model 11 spectrophotometer.

4. Culture tubes are consistently freed of biologically effective traces of impurities when they are treated with chromic-sulfuric acid, and subsequent

washings are terminated with at least one 20 minute period of heating in water at 120°.

5. The range of variations in bacterial growth occurring among identically charged tubes can be reduced to about one-third by the use of a constant temperature water bath in place of a convection-type air incubator. In a water bath under optimal conditions, growth variations are less than twice as large as the variations in readings imposed by instrumental precision.

6. Refrigerator storage of agar cultures or of washed organisms grown in liquid cultures results in a slow linear decrease in the amount of growth induced in a standard medium by inocula taken from the stored cultures.

7. When organisms from freshly grown cultures serve as the inoculum, the ensuing growth is the same under standard conditions, regardless of the age of the parent culture of the inoculum.

8. Ordinary variations in drop size occurring in single drop inoculation are negligible as sources of experimental error.

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# THE INHIBITORY EFFECTS OF DL-ALANINE, L-GLUTAMIC ACID, L-LYSINE, AND L-HISTIDINE ON THE ACTIVITY OF INTESTINAL, BONE, AND KIDNEY PHOSPHATASES\*

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Glycine and other  $\alpha$ -amino acids in very low concentration increase the activity of tissue phosphatases, whereas there have been indications that in higher concentrations they appear to decrease it (1). It was recently shown that glycine, in concentrations higher than the 0.006 M necessary for optimal activity, inhibited the action of bone and intestinal phosphatases, the former in an entirely non-competitive manner and the latter, largely so (2). The inhibition was found to be dependent on the presence of the free carboxyl and amino groups of glycine. Esterification of the carboxyl group reduced the inhibition to about one-half the value obtained with glycine and the introduction of methyl groups into the amino group decreased the inhibition even more markedly.

These findings naturally raised the question as to the extent to which other  $\alpha$ -amino acids might inhibit phosphatase activity. The present paper is concerned chiefly with the study of the inhibitory effects of DL-alanine, of the basic amino acids, L-lysine and L-histidine, and of the dicarboxylic L-glutamic acid on the activity of intestinal, bone, and kidney phosphatases.

## EXPERIMENTAL

The methods for preparing dialyzed phosphatase extracts and for measuring their activity have been described previously in detail (3). In most of the experiments reported in the present paper, the concentration of enzyme preparation was 12.5 per cent by volume of the hydrolysis mixture; that of the substrate, sodium  $\beta$ -glycerophosphate, was in the maximal range, 0.0127 or 0.0254 M. The concentration of the buffer, sodium diethyl barbiturate, was 0.5 gm. per 100 cc. of hydrolysis mixture or 0.024 M. In accordance with considerations previously presented (3), concentrations of 0.01 M magnesium ion and 0.006 M glycine were present in order to obtain optimal enzyme activity. Each determination of phosphatase activity was

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carried out at optimal alkaline pH, assured by running a series of hydrolyses constituting a very closely spaced pH-activity curve in and about the optimal range. The reaction velocities were determined from the amount of phosphorus liberated as inorganic phosphate per cc. of hydrolysis mixture at three time intervals, spaced as equally apart as possible, during the zero order portion of the hydrolysis and were expressed as micrograms of phosphorus per cc. per minute. The liberated inorganic phosphate was determined by the method of Fiske and Subbarow (4) after assurance had been gained that the colorimetric readings were not affected by the concentrations of the  $\alpha$ -amino acids employed. All hydrolyses were conducted at 25° in a water thermostat regulated to within 0.01–0.05°. The  $\alpha$ -amino acids were of reagent grade and were from the following sources: Fisher, Eastman Kodak, Pfanstiehl, and Merck.<sup>1</sup> The  $\alpha$ -amino acids were made up in solutions which were adjusted to about pH 9.0. For any particular series of experiments designed to characterize the inhibitory effect of a particular amino acid on a phosphatase preparation, reaction velocities were usually determined in the presence of from four to seven different concentrations of the  $\alpha$ -amino acid. Several bone and intestinal and one kidney phosphatase preparations were used in the course of this work. Other techniques are described in connection with specific experiments.

### Results

*Reversibility of  $\alpha$ -Amino Acid Inhibition*—In the course of earlier work (1) it was pointed out that intestinal and kidney tissues have considerable autolytic capacity and that their crude extracts, therefore, probably contain relatively high concentrations of proteolytic products, including  $\alpha$ -amino acids. Observations on the relationship between the concentrations of such preparations and their rates of reaction as well as the finding that dialysis increased their phosphatase activity indicated that the inhibition by proteolytic products was reversible. However, more definite proof is offered in Table I. It may be seen that a concentration of 0.12 M DL-alanine in the final hydrolysis mixture reduced the phosphatase activity of an intestinal preparation to 25 to 31 per cent of its original activity. Dialysis resulted in an increase of the enzyme activity; so that at the end of 24 to 28 hours it was 73 to 83 per cent of the activity of a sample of the uninhibited phosphatase preparation which had been dialyzed under similar conditions. Again, a concentration of 0.0025 M L-histidine decreased the phosphatase activity to 20 per cent of the uninhibited activity; dialysis restored it to 60 per cent.

<sup>1</sup> We are indebted to the Department of Biochemistry, Cornell University Medical College, for a sample of L-lysine.

*Expression of Degree of Inhibition by  $\alpha$ -Amino Acids*—It was previously shown that the inhibition of bone phosphatase by glycine was non-competitive (2). The extent of such inhibition may be expressed in accordance with the following transposition of the Michaelis-Menten equation (5, 6)

$$K'_I = \frac{I}{\frac{V_0}{V} - 1} \quad (1)$$

where  $V_0$  is the reaction velocity in the absence of any inhibitor and  $V$  is the velocity at the concentration,  $I$ , of the inhibitor.  $K'_I$ , the dissociation

TABLE I

*Reversal of Amino Acid Inhibition of Phosphatase Activity*

A rat intestinal phosphatase was mixed with a solution of the amino acid or an equivalent amount of distilled water. The initial activities of the phosphatase-water and phosphatase-amino acid mixtures were determined and each mixture then dialyzed against several changes of distilled water. Samples were drawn from dialysis bags at the times shown and the activities determined. Test hydrolyses were run at 25° with 0.0254 M sodium  $\beta$ -glycerophosphate, optimal pH, magnesium, and glycine concentrations. Corrections made for dilution of contents of dialysis bags.

Experiment No.	Duration of dialysis	Activity; P liberated as inorganic phosphate per cc. per min. in		Ratio of activity of enzyme-amino acid mixture to that of enzyme-water mixture
		Enzyme-water mixture	Enzyme-amino acid mixture	
	<i>hrs.</i>	$\gamma$	$\gamma$	<i>per cent</i>
1. Initial concentration of 0.48 M DL-alanine diluted to 0.12 M in test; dialysis at room temperature	0	0.92	0.23	25
	3½	0.81	0.20	36
	24	0.66	0.48	73
2. Initial concentration of 0.48 M DL-alanine diluted to 0.12 M in test; dialysis at ice box temperature	0	0.89	0.28	31
	28	0.77	0.64	83
3. Initial concentration of 0.021 M L-histidine diluted to 0.0025 M in test; dialysis at ice box temperature	0	0.76	0.15	20
	71	0.67	0.40	60

constant of the assumed enzyme-inhibitor complex, is independent of the substrate concentration. The inhibition of intestinal phosphatase by glycine is largely, though not entirely, non-competitive in type (2). In the range of near maximal or maximal substrate concentrations, 0.0127 to 0.0254 M sodium  $\beta$ -glycerophosphate, and at concentrations of glycine greater than about 0.03 M, the inhibition is usually over 95 per cent non-competitive in type.



Although it was not feasible to establish, as precisely as had been done for glycine, the extent of non-competitive inhibition for each  $\alpha$ -amino acid and each tissue phosphatase employed, a few experiments indicated that, at the higher substrate concentrations used in this study, the inhibition was either completely or practically non-competitive and that, therefore, Equation 1 might be used to define the extent of such inhibition. It was found, for example, that DL-alanine inhibited bone phosphatase entirely in a non-competitive manner and that the inhibition of intestinal phosphatase activity by DL-alanine, L-glutamic acid, and L-histidine was non-competitive at the higher concentrations of substrate, 0.0127 to 0.0254 M sodium  $\beta$ -glycerophosphate.

TABLE II

*Inhibitory Effect of Glycine on Activity of Phosphatases*

Reaction velocities determined under optimal conditions and at 0.0127 M sodium  $\beta$ -glycerophosphate, except for preparation RIL-d, in which the concentration was 0.0254 M. Extent of inhibition expressed in accordance with Equation 1 (see the text):  $K'_i = I/((V_0/V) - 1)$ . For each  $K'_i$  value, reaction velocities determined at six to nine concentrations of glycine.

Source	Preparation	Dissociation constant, $K'_i$ <i>mole per l.</i>
Intestinal phosphatase, rat .....	RIK-d	0.050
“ “ “ .....	RIL-d	0.060
“ “ human .....	MID-d	0.075
“ “ “ .....	“	0.047
Bone phosphatase, cat .....	CaBA-d	0.049
“ “ “ .....	“	0.035
“ “ rat .....	RBM-d	0.039

The value of  $K'_i$  was obtained by plotting the relative reaction velocities, that is, the velocities expressed as per cent of the velocity in the absence of inhibiting amino acid, against the negative logarithms of the concentrations of the amino acid. From the curves thus obtained, which were linear in form except at the extreme upper and lower limits,  $K'_i$  was readily determinable, in accordance with Equation 1, as that concentration of amino acid which gave a reaction velocity equal to 50 per cent of the velocity in the absence of inhibitor. The units of  $K'_i$  are moles per liter.

*Inhibitory Effects of Glycine and DL-Alanine*—It was previously found (2) that the value of  $K'_i$  for the inhibitory action of glycine on a rat bone phosphatase, preparation RBM-d, was 0.039. In Table II are shown additional determinations of the inhibitory effect of glycine on cat bone phosphatase and on human and rat intestinal phosphatases. There was

some variation in the values for  $K'$ , which may in part have been due to the source of glycine, since with the same bone phosphatase, CaBA-d, and with the same intestinal phosphatase, MID-d, the Pfanstiehl product gave a somewhat lower value than did the Eastman product. There appeared to be no significant difference between the values for  $K'$ , for the intestinal preparations and those for the bone preparations. The average value of  $K'$ , for the inhibitory effect of glycine in these seven series of experiments on five phosphatase preparations was 0.051 (s.d. = 0.013).

Table III shows the results of eight series of experiments on the effects of DL-alanine on the activity of cat bone, rat bone, rat intestinal, human intestinal, and rat kidney phosphatases. The values of  $K'$ , for the bone

TABLE III

*Inhibitory Effect on DL-Alanine on Activity of Phosphatases*

Conditions and calculations as described in Table II. Concentration of sodium  $\beta$ -glycerophosphate 0.0127 M, except where noted. For each  $K'$  value, reaction velocities determined at four to seven concentrations of DL-alanine.

Source	Preparation	Dissociation constant, $K'$
		<i>mole per l.</i>
Intestinal phosphatase, human.....	MID-d	0.107
“ “ “.....	“	0.096
“ “ rat.....	RIL-d	0.144*
“ “ “.....	“	0.132
Bone phosphatase, cat.....	CaBA-d	0.090
“ “ “.....	“	0.124
“ “ rat.....	RBM'-d	0.096*
Kidney phosphatase, rat.....	RKA-d	0.076*

\* Concentration of sodium  $\beta$ -glycerophosphate 0.0254 M.

phosphatases did not appear to differ significantly from those for the intestinal phosphatases. The average value of  $K'$ , for the bone and intestinal phosphatase inhibition by DL-alanine was 0.113 (s.d. = 0.021). The value of  $K'$ , for one series of experiments on the inhibition of rat kidney phosphatase by DL-alanine was 0.076.

*Inhibitory Effects of L-Glutamic Acid, L-Lysine, and L-Histidine*—In order to explore further the inhibitory effects of  $\alpha$ -amino acids, it was decided to study the dicarboxylic L-glutamic acid and the two basic amino acids, L-lysine and L-histidine. Table IV shows, first, that the inhibitory effects of L-glutamic acid on rat intestinal phosphatase, of L-lysine on rat bone and kidney phosphatases, and of L-histidine on intestinal, bone, and kidney phosphatases are of a higher order of magnitude than those of glycine or DL-alanine on these enzymes. Secondly, there is a distinct difference

between the magnitude of these effects on bone and kidney phosphatases, on the one hand, and those on intestinal phosphatase, on the other. Thus, the  $K'_i$  values, 0.030, for the inhibition of three different rat intestinal phosphatases by glutamic acid were lower than those, 0.088 to 0.129, for rat bone or rat kidney phosphatases. For the inhibition by the two basic amino acids, L-lysine and L-histidine, the situation was reversed, the values of  $K'_i$  being definitely higher for the intestinal preparations than for the kidney or bone phosphatases. For example, the values of  $K'_i$  for the

TABLE IV

*Inhibitory Effects of L-Glutamic Acid, L-Lysine, and L-Histidine on Activity of Phosphatases*

Conditions and calculations as described in Table II. Concentration of sodium  $\beta$ -glycerophosphate 0.0254 M, except where noted. For each  $K'_i$  value, reaction velocities were determined at three to five concentrations of amino acid. Rat tissues were used.

Source	Preparation	Dissociation constant, $K'_i$		
		L-Glutamic acid	L-Lysine	L-Histidine
		<i>mole per l.</i>	<i>mole per l.</i>	<i>mole per l.</i>
Intestinal phosphatase	RIM-d	0.030	0.141	0.0064
	RIK-d	0.030*		
	RIO-d	0.030	0.085	
	RIN-d		0.059	
	RIL-d			0.0063
Bone phosphatase	"			0.0060*
	RBN-d	0.100	0.0105	0.0022
	RBM'-d		0.0091	0.0034
	RBO-d	0.129		
Kidney phosphatase	RBM-d	0.088		
	RKA-d	0.100	0.0093	0.0028

\* Concentration of sodium  $\beta$ -glycerophosphate 0.0127 M.

inhibition of three different rat intestinal preparations by L-lysine were 0.06 to 0.14, whereas the values for the inhibition of the two bone and one kidney phosphatases were 0.009 to 0.010. Most striking of the inhibitory effects obtained were those by L-histidine. The values of  $K'_i$  for this amino acid were 0.006 for rat intestinal phosphatases and 0.002 to 0.003 for bone and kidney phosphatases. The differences between the action of L-glutamic acid and L-histidine on a rat intestinal phosphatase, RIM-d, and a rat bone phosphatase, RBN-d, as well as the magnitudes of these effects, are illustrated in Fig. 1, where the relative reaction velocities at the various concentrations of the amino acids are shown.

**Possible Role of Incidental, Non-Phosphatasic Components on Inhibitory Effects of Amino Acids**—The possibility existed that the inhibitory effects observed might be caused by the presence of traces of heavy metal ions, such as mercuric or cupric, in the amino acids. Saturation with hydrogen sulfide of solutions of 1.0 M DL-alanine, 1.0 M glycine, 0.23 M L-histidine, or 0.35 M L-lysine resulted in no or only very faint darkening of the solutions. These concentrations of amino acids were much higher than those which gave appreciable inhibition. Yet concentrations of 0.00125 M mercuric or cupric ions which, as shown previously (3) or in the course of this work, inhibited phosphatase activity only about 10 to 20 per cent yielded intensely

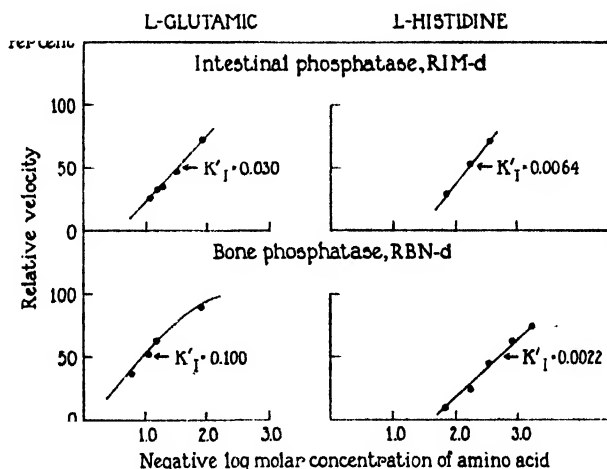


FIG. 1. Relative activities of bone and intestinal phosphatases in presence of varying concentrations of L-histidine and L-glutamic acid. The dissociation constant,  $K'_1$ , of the phosphatase-amino acid complex is equal to  $1/((V_0/V) - 1)$  and represents that concentration of amino acid at which 50 per cent inhibition occurs.

dark suspensions of the metallic sulfides on saturation with hydrogen sulfide. Moreover, concentrations of  $1.25 \times 10^{-6}$  M mercuric and cupric ions which gave faint but definite darkening on saturation with hydrogen sulfide exerted no inhibitory effect on phosphatase activity. There was therefore little likelihood that at the concentrations of amino acids at which inhibition occurred enough mercuric or cupric ion was present to account for any portion of this inhibition.

The possibility also existed that the extent of inhibition was influenced by the presence of substances in the phosphatase preparations other than the active phosphatasic components. It might be conceived, for example, that intestinal phosphatase was not inhibited by L-lysine to the same extent as bone or kidney phosphatase because there was some protein or other

component in the intestinal phosphatase preparation which interacted with this amino acid and thus reduced the amount available for interaction with the active enzyme. Although rigorous exclusion of this possibility can only be achieved by isolating each tissue phosphatase in pure form, indirect methods may be used to determine whether non-phosphatase substances present in these tissue extracts are responsible to any significant degree for the characteristic inhibition of the various tissue phosphatases. These methods are similar to those previously employed (3) to show that

TABLE V

*Effect of Adding Heat-Inactivated Tissue Phosphatase Preparation on Inhibition of Phosphatase Activity by L-Lysine*

Tissue phosphatase preparations were heated to 70° for  $\frac{1}{2}$  hour to destroy enzyme activity and then added to the active phosphatase preparation as shown in the table. Concentration of L-lysine in final hydrolysis mixture 0.0123 M for RIN-d and RBN-d, 0.0183 M for RIM-d, and 0.0108 M for RKA-d. Concentration of sodium  $\beta$ -glycerophosphate, 0.0254 M, and other conditions optimal, as described in the text.

Source	Active phosphatase	Inactivated phosphatase	Reaction velocity		
			As P liberated as inorganic phosphate per cc. per min.		As fraction of uninhibited velocity
			No lysine	Lysine present	
			$\gamma$	$\gamma$	per cent
Intestinal preparation, active	12.5% RIN-d	None	1.35	1.02	76
	12.5% "	12.5% RBN-d	1.19	0.95	80
	12.5% RIM-d	None	0.82	0.70	85
	12.5% "	12.5% RBN-d	0.80	0.64	80
Bone preparation, active	12.5% RBN-d	None	0.90	0.42	47
	12.5% "	12.5% RIN-d	0.91	0.43	47
Kidney preparation, active	12.5% RKA-d	None	1.42	0.84*	59
	12.5% "	12.5% RIO-d	1.44	0.81	56

\* Extrapolated from inhibition curve of rat kidney preparation.

the inhibition of bone and kidney phosphatases by bile acids and the lack of inhibition of intestinal phosphatase were not dependent on the presence of non-phosphatase components in these preparations.

Table V shows that 0.0123 M L-lysine reduced the activity of rat intestinal phosphatase, RIN-d, to essentially the same extent, about 80 per cent of the uninhibited velocity, whether heat-inactivated rat bone phosphatase was present or absent. On the other hand, this concentration of L-lysine reduced rat bone or rat kidney phosphatase much more markedly, to about 50 per cent of the uninhibited velocity, and this degree of inhibi-

tion was not affected by the presence of heat-inactivated intestinal phosphatase.

If non-phosphatasic components do not appreciably influence the inhibition, then a mixture of two phosphatases, each of which is inhibited to a characteristic degree by a given amino acid, should be inhibited to an extent which is the mathematical resultant of each of these inhibitions. Three such experiments are shown in Table VI. To illustrate, the reaction velocities of a 12.5 per cent concentration of rat intestinal phosphatase, RIN-d, as may be seen from Table V, were 1.35  $\gamma$  of P per cc. per minute without inhibitor and 1.02  $\gamma$  of P per cc. per minute in the presence of 0.0123 M L-lysine. The corresponding values for a 12.5 per cent concentration of rat bone phosphatase, RBN-d, were 0.90  $\gamma$  and 0.42  $\gamma$  of P per cc. per minute,

TABLE VI

*Inhibitory Effect of L-Lysine on Activity of Mixtures of Different Tissue Phosphatases*

Activities determined under optimal conditions as described in the text. Concentration of sodium  $\beta$ -glycerophosphate 0.0254 M. Calculations of expected velocities described in the text.

Composition of mixtures	Concentration of L-lysine	Reaction velocity as P liberated per cc. per min.				As fraction of uninhibited velocity	
		In absence of lysine		In presence of lysine			
		Calculated	Observed	Calculated	Observed	Calculated	Observed
	M	$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent	per cent
12.5% RIN-d + 12.5% RBN-d.....	0.0123	2.25	2.02	1.44	1.33	65	66
6.25% RIM-d + 6.25% RBN-d.....	0.0083	0.86	0.81	0.64	0.57	74	70
6.25% RIO-d + 6.25% RKA-d.....	0.0108	1.20	1.07	0.87	0.73	72	68

respectively. The *calculated* reaction velocities of a mixture containing 12.5 per cent of each of these phosphatases were  $1.35 + 0.90$  or  $2.25 \gamma$  of P per cc. per minute in the absence of inhibitor and  $1.02 + 0.42$  or  $1.44 \gamma$  of P per cc. per minute in the presence of 0.0123 M L-lysine, or 65 per cent of the *calculated* uninhibited reaction velocity. The reaction velocities observed for the mixture were  $2.02 \gamma$  of P per cc. per minute in the absence of, and  $1.33 \gamma$  of P per cc. per minute, or 66 per cent of the uninhibited *observed* value, in the presence of L-lysine. In this instance, as in the others shown in Table VI, there was fairly good agreement, within experimental error, between the observed reaction velocities of the phosphatase mixtures and the velocities calculated on the basis that L-lysine exerted its characteristic inhibitory effect on each of the active phosphatase components and that this effect was not significantly influenced by the presence of non-phosphatasic components.

## DISCUSSION

The present investigation has shown that a number of  $\alpha$ -amino acids inhibit bone, kidney, and intestinal phosphatases. There is evidence to indicate that inhibition of enzyme activity by  $\alpha$ -amino acids may be a more general phenomenon. Abderhalden, Rindtorff, and Schmitz (7) noted that  $\alpha$ -amino acids inhibited tryptic and ereptic activity, although this observation may, of course, be explained in terms of the retardant effect which reactant products generally exert. However, Edlbacher and Zeller (8) found that glycine, alanine, and lysine inhibited arginase activity and Girsavicius, Efendi, and Ryzhova (9) reported that the enzymic conversion of methylglyoxal to lactic acid was inhibited by concentrations of approximately 0.02 M tryptophan, glutamic acid, aspartic acid, arginine, and histidine, but that similar concentrations of glycine, alanine, and leucine were without effect. More recently, Hunter and Downs (10) found that the action of arginase upon arginine at pH 8.4 was inhibited by all  $\alpha$ -amino acids of the naturally occurring L configuration, but not by D- $\alpha$ -amino acids, amino acids having the amino group in other than the  $\alpha$  position, urea, or native protein.

In studying the mechanism of the inhibition of bone and intestinal phosphatases by glycine, Bodansky (2) observed that the inhibition was dependent on the presence of the free carboxyl and amino groups of glycine. Glycine ethyl ester inhibited about only half as much as glycine, and monomethyl- and dimethylglycine had still less inhibitory effect. These findings as well as those reported in the present paper indicate an interaction between the amino acid and the presumably protein phosphatase. Although it is known that proteins as well as  $\alpha$ -amino acids may interact with each other (11, 12), and although it is possible that amino acids may interact with some essential metallic component of the enzyme protein, there appears to be no study in the literature of the interaction of a protein with an amino acid which would serve to describe the nature of the interactions between phosphatase and the amino acids which result in inhibition.

The observations reported in the present work raise the possibility that the stream of protein metabolism, either normal or deranged, may affect the enzymic activities of the organism *in vivo*. There are, however, at present practically no data to support or deny this possibility. A. Bodansky (13) observed that, in contrast to the rise in the activity of serum phosphatase after ingestion of carbohydrate in dogs, there was a decrease after the ingestion of protein (meat). A number of workers (14-16) have reported that additions of large amounts of various  $\alpha$ -amino acids to the diet produced toxic effects in the rat, but no relationship of such effects to inhibition of enzyme activity has been adduced or even suggested.

Of interest is the finding that, although glycine and DL-alanine inhibit rat bone and intestinal phosphatases to about the same extent, the basic amino acids, L-histidine and L-lysine, exert a greater inhibitory effect on rat bone phosphatase than on intestinal phosphatase, whereas the reverse holds for the dicarboxylic acid, L-glutamic acid. Whether this relationship with basic and acidic amino acids is more generally applicable to phosphatase remains to be determined. It may also be noted that in the series of experiments here reported with one rat kidney phosphatase preparation the degree of inhibition obtained with the various  $\alpha$ -amino acids resembled the degrees of inhibition of bone phosphatase more closely than those of intestinal phosphatase. It was previously reported (3) that the inhibitions of bone and kidney phosphatases by 0.006 M bile acids were of the same order of magnitude, whereas intestinal phosphatases were not inhibited by bile acids.

The results of Hunter and Downs (10) on the inhibition of arginase activity by various amino acids may be examined with respect to those amino acids which were also used in our investigation. At the highest concentration, 0.089 M, of the substrate arginine, 50 per cent inhibition was produced by 0.060 M L-lysine. Glycine proved to be one of the poorest inhibitors; at an arginine concentration of 0.0223 M, as much as 5.0 M glycine was necessary to produce an inhibition of 50 per cent. L-Histidine which was found in the present investigation to be a very potent inhibitor of bone, kidney, and intestinal phosphatases was a very poor inhibitor of arginase activity. In contrast to the concentrations of 0.002 to 0.003 M which produce a 50 per cent inhibition of bone and kidney phosphatases, Hunter and Downs' results show that a concentration of 3.8 M L-histidine was necessary to produce this degree of inhibition of arginase activity.

#### SUMMARY

1. The inhibitory effects of DL-alanine, L-lysine, L-glutamic acid, and L-histidine on rat bone, kidney, and intestinal phosphatases have been investigated and have been evaluated in terms of the dissociation constants of the assumed, dissociable amino acid-phosphatase complexes. The inhibitory effects of L-histidine were particularly marked; an inhibition of 50 per cent was produced by 0.006 M of this acid on rat intestinal phosphatase and by 0.002 to 0.003 M on rat bone and kidney phosphatases.

2. The inhibition of phosphatase by  $\alpha$ -amino acids, as illustrated in the instances of DL-alanine and L-lysine, may be largely reversed through dialysis.

3. The basic amino acids, L-histidine and L-lysine, exerted a greater inhibitory effect on rat bone and kidney phosphatases than on intestinal phosphatase, whereas the reverse held for the dicarboxylic L-glutamic acid.



4. It was shown that, as illustrated in the instance of L-lysine, the characteristic inhibitions of the various tissue phosphatases were not due to or influenced by the presence of non-phosphatasic components present in these enzyme preparations.

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## DIMETHYLTHETIN AS A BIOLOGICAL METHYL DONOR\*

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In our earlier work (1) on a study of the ability of choline and betaine to support the growth of young rats on a homocystine-containing, methionine-free diet, we began to investigate the specificity of choline and betaine with respect to this ability. The promotion of growth under these conditions was interpreted as indicating a synthesis of methionine from homocystine by an actual transfer of the methyl group, an interpretation borne out by tracing the methyl group with deuterium (2).

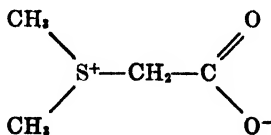
By the growth criterion the methyl-donating ability of systematically varied groups of compounds structurally related to choline was studied (3). Analogues of choline were tested in which one or two of the N-methyl groups had been substituted by N-ethyl groups. Other analogues were used in which various substitutions had been made in the ethanol moiety. The arsenic analogue of choline was also tested as well as various betaines and related compounds. The high degree of specificity with regard to structure in relation to the ability to act as a methyl donor in this connection was shown by the fact that, of the many compounds tested, only choline (and simple derivatives such as lecithin and phosphorylcholine), betaine, and dimethylethylhydroxyethylammonium chloride were found to support growth under these conditions (3).

Of particular interest was the finding that arsenocholine, although capable of preventing perosis in the chick (4) and hemorrhagic kidney (5) and fatty infiltration of the liver in the rat (6), was incapable of supporting growth of the white rat on the methionine-free, homocystine-containing diet (6, 3). Evidently the methyl groups of arsenocholine either were not labile or were not being transferred at a rate fast enough to form methionine in sufficient amounts to support growth.

In a continuation of these investigations we extended the work to the sulfur analogue of betaine referred to in the literature as sulfobetaine or di-

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methylthetin. The first test of this compound<sup>1</sup> as a source of labile methyl groups involved a diet containing a hydriodic acid hydrolysate of casein (3) as a source of an amino acid mixture deficient in methionine. The positive



results we obtained were announced in a Harvey lecture (7) on transmethylation.<sup>2</sup>

More recently we have tested dimethylthetin as a source of labile methyl groups in growth experiments with white rats on a mixture of the various amino acids which did not contain methionine but to which homocystine had been added. The experimental procedures used and the results obtained from these latter experiments are reported in this communication.

The animals which received the basal diet without added supplement of a methyl donor did not grow and in several instances died before the experiment was terminated. However, when either choline or betaine was added to the diet growth ensued. The rats which received dimethylthetin grew at a rate comparable to those receiving the choline or betaine. It is therefore concluded that this methylsulfonium compound, like the latter compounds, can provide a source of biologically labile methyl groups. The biological and chemical implications of this are being investigated.

#### EXPERIMENTAL

Dimethylthetin chloride was prepared by treating chloroacetic acid with dimethyl sulfide according to the method used by Brown and Letts (9) in the preparation of dimethylthetin bromide. The analytical data and the properties of the compound agreed with those recorded in the literature.

Male white rats weighing about 100 gm. were allowed to grow for several days on Diet I which had the following percentage composition: amino acid mixture 21.3 (10), cystine 0.4, Osborne and Mendel salt mixture (11) 4.0, sucrose 53.3, hydrogenated vegetable oil 19.0, corn oil 1.0, and DL-methio-

<sup>1</sup> A part of the dimethylthetin used in this test was placed at our disposal through the generosity of Dr. A. D. Welch, who had earlier found that this compound possessed both antihemorrhagic and lipotropic activities to which we referred in a previous communication (3).

<sup>2</sup> As this manuscript was in the last stages of completion, the report of Dubnoff and Borsook (8) confirming that dimethylthetin can function as a methyl donor came to hand. Through *in vitro* studies with a liver homogenate they found that this sulfonium compound was a highly efficient methylating agent in the conversion of homocysteine to methionine.

nine 1.0. The fat-soluble vitamins were dissolved in the corn oil and supplied in the following quantities for each kilo of diet: vitamin A 7200 units, vitamin D 1200 units, menadione 1.0 mg., and  $\alpha$ -tocopherol acetate 40 mg. The water-soluble vitamins were present in the diet in the following amounts for each kilo of diet: thiamine chloride, riboflavin, pyridoxine hydrochloride, nicotinic acid, and *p*-aminobenzoic acid 10.0 mg. each, calcium pantothenate 50 mg., inositol 100 mg., pteroylglutamic acid (Folvite) 1.0 mg., and biotin 0.1 mg. The rats were then placed on Diet II, which was identical with

TABLE I  
*Food Consumption and Growth Changes*

Group No.	Rat No.	Diet No. and supplement	Days on diet	Average daily food consumption	Starting and final weights	Average daily weight change
				gm.	gm.	gm.
I	2963	II	19	7.3	94-80	-0.7
	2964	"	7	3.9	92-88	-0.6
	2966	"	6	5.7	130-121	-1.5
	2967	"	3	2.3	141-133	-2.7
	2970	" + choline	17	11.8	80-179	+5.3
	2961	" + dimethylthetin	20	6.8	86-128	+2.1
	2965	" + "	20	9.4	127-204	+3.8
II	2973	"	27	6.9	130-134	+0.1
	2976	"	27	6.6	115-114	0.0
	2978	"	27	5.7	135-109	-0.9
	2979	" + choline	28	10.2	121-205	+3.0
	2984	" + "	28	10.9	103-180	+2.7
	2971	" + betaine	28	6.0	90-124	+1.2
	2975	" + "	28	12.0	132-208	+2.7
	2980	" + "	28	10.6	143-220	+2.7
	2972	" + dimethylthetin	28	9.3	125-174	+1.8
	2974	" + "	27	6.9	110-123	+0.5
	2977	" + "	28	12.5	135-184	+1.8

Diet I, except that DL-homocystine (0.87 per cent) replaced the DL-methionine. The methyl compound to be tested was added to Diet II by substitution for an equal weight of sucrose. The first group of rats (Nos. 2961, 2963 to 2967, 2970) was placed on the homocystine diet (Diet II) without supplementation for 7 days, after which they were fed the supplements indicated in Table I. The second group of rats (Nos. 2971 to 2980, 2984) was transferred directly from Diet I to the diets indicated in Table I. The growth rate and food consumption data are given in Table I. The supplements were added to the diet in the following amounts: choline chloride 0.5, betaine chloride 0.55, and dimethylthetin chloride 0.84 per cent.

## SUMMARY

Dimethylthetin plus homocystine have been shown to be able to replace methionine in the diet of the white rat. The resulting growth was comparable to that obtained with choline, or betaine, plus homocystine. It has therefore been concluded that this sulfonium compound, like choline and betaine, can serve as a source of labile methyl groups in the diet.

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# INTERACTION BETWEEN TOBACCO MOSAIC VIRUS AND BOVINE SERUM ALBUMIN\*

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During the initial phase of a study of the rate of sedimentation of tobacco mosaic virus in bovine serum albumin solutions, carried out because of the potential importance of results obtained by Sharp *et al.* (1) with influenza virus, it was observed that tobacco mosaic virus was precipitated from its solution in 0.1 M phosphate buffer at pH 7 when solid serum albumin was added. Upon further examination, it was found that the virus would remain in solution after the addition of serum albumin if the pH was about 4.5 or 4.7. At pH 4.5, tobacco mosaic virus is negatively charged and serum albumin is positively charged, while above about pH 4.8 both are negatively charged.

Because of this observation, the author thought it worth while to study the interaction between tobacco mosaic virus and bovine serum albumin in greater detail. In a preliminary communication (2), it was reported that tobacco mosaic virus was precipitated in crystalline form by serum albumin under conditions of like charge, either positive or negative, except when the ionic strength of the ordinary electrolytes also present was too low. It was also reported that under conditions of opposite charge a precipitate was formed at very low values of the ionic strength of the ordinary electrolytes. This latter behavior was regarded as being consistent with the results obtained by Kleczkowski (3) in his studies on the mutual precipitation of tobacco mosaic virus and various proteins under conditions of opposite charge.

The present communication consists of a detailed report of the studies on the interaction between bovine serum albumin and tobacco mosaic virus, with particular emphasis upon the precipitation of the virus under conditions of like charge.

## *Materials and Methods*<sup>1</sup>

The virus preparation used in this study was obtained by the method of differential centrifugation from the juice of Turkish tobacco plants

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† Contribution No. p2-48 from the Department of Physics of the University of Pittsburgh.

<sup>1</sup> The author wishes to acknowledge the technical assistance of Miss Harriet L.

diseased with the Rockefeller Institute strain of tobacco mosaic. The final product contained 21 mg. per cc. of virus and 1 part per 100,000 of phenyl mercuric nitrate in a phosphate buffer at pH 7. The bovine serum albumin was obtained from Armour and Company.

A series of stock buffers of 0.2 ionic strength was prepared as follows: for pH values between 2.0 and 4.0, chloroacetic acid and sodium hydroxide were used; for pH values between 4 and 6, sodium acetate and acetic acid were used; for pH values between 7 and 8.5, sodium hydrogen phosphate and sodium dihydrogen phosphate were used; and for pH values between about 9 and 10, borax and either hydrochloric acid or sodium hydroxide were used.

Measurements of pH on the buffers and on the serum albumin-virus systems were made with a Beckman pH meter. Potassium acid phthalate at a concentration of 0.05 M, which has a pH of 4.000, was used as a reference. Nitrogen analyses were made by a colorimetric modification of the Kjeldahl method, as described by Miller (4).

### *Results*

A series of virus-serum albumin systems was set up in which an attempt was made to maintain all variables constant except pH. Each was contained within a 15 cc. glass centrifuge tube and was composed of 0.5 cc. of stock buffer, 0.05 gm. of serum albumin, 0.15 cc. of stock virus solution, and 0.3 cc. of water. Thus, the serum albumin and the virus concentrations were approximately 5 and 0.3 per cent by weight, respectively, while the ionic strength was essentially constant with an approximate value of 0.1. The results obtained are shown in Table I. Because precipitation was apparently very slow in some systems and apparently very rapid in others, it was relatively difficult to find a wholly objective means of evaluating the solubility of tobacco mosaic virus in serum albumin solutions. It was decided to compare the systems after a constant arbitrary period of time, namely 24 hours. The results in Table I indicate that, except for pH values between about 4 and 5, tobacco mosaic virus was insoluble in 5 per cent serum albumin solutions with ionic strength of 0.1. The turbidities obtained at pH 3.6 and 4 are not necessarily indications of interaction between serum albumin and tobacco mosaic virus, because the virus is insoluble at these pH values even in the absence of serum albumin (5). However, at pH values approximating 2.6, the virus is soluble in aqueous media with very low ionic strength. Accordingly, an experiment

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Carnelly and Miss Martha M. Raup. The author is indebted to Dr. Marie Fischer for the preparation of the virus.

was carried out at about pH 2.6 in a chloroacetic acid buffer with an ionic strength of 0.02. The same virus concentration was used as in the preceding experiment, but the serum albumin concentration was varied. The results are listed in Table II. It was found that 1 per cent serum albumin caused no immediate effect, but upon standing 24 hours, a small amount of precipitate was formed. With 3 per cent and more of serum albumin, apparently complete precipitation of the virus took place. In order to carry out this experiment and maintain the pH essentially con-

TABLE I

*Solubility of Tobacco Mosaic Virus in 5 Per Cent Serum Albumin Solutions at Ionic Strength of 0.1 and at Various pH Values*

Buffer	pH of buffer	pH of serum albumin and virus in buffer	Appearance immediately	Appearance after 24 hrs.
Chloroacetate	2.02		Turbid	Gel
"	2.95	3.62	"	Turbid
Acetate	3.92	4.02	"	"
"	4.20	4.37	Clear	Clear
"	4.40	4.58	"	"
"	4.60	4.78	"	"
"	4.80	4.92	"	"
"	4.95		"	Turbid (thixotropic)
"	5.01	5.15	Turbid	" "
"	5.21	5.31	"	Ppt.
"	5.41		"	"
"	5.65		"	"
"	5.81		"	"
Phosphate	5.93		"	"
"	6.97		"	"
"	7.93		"	"
"	8.89		"	"
Borate	10.35		"	"

stant, it was necessary to titrate the serum albumin to pH 2.6 before mixing it with the virus and buffer. Thus, it can be seen that tobacco mosaic virus could be precipitated from solution by serum albumin when the pH was either alkaline to the isoelectric points of both virus and serum albumin or acidic to the isoelectric points of both. However, in media with ionic strength of 0.1, no precipitation not attributable to the normal insolubility of tobacco mosaic virus was observed when the pH was intermediate between the pH values of the isoelectric points of virus and serum albumin.

Additional experiments, similar to those at pH 2.6, were carried out at pH 4.4, 5.2, and 5.8. In each, pH, ionic strength, and virus concentra-



tion were maintained constant, but serum albumin concentration was varied. The results are also shown in Table II. At pH 4.4, when the ionic strength was held at the value of 0.1, there was no precipitation even when the serum albumin concentration reached 35 per cent. At pH 5.2 and ionic strength 0.1, precipitation within a 24 hour period occurred with 3 per cent but not with 2 per cent serum albumin. At pH 5.8 and the same ionic strength, evidence of precipitation at the end of 24 hours was obtained with 2 per cent serum albumin.

Tobacco mosaic virus concentration and pH were kept essentially con-

TABLE II  
*Effect of Varying Serum Albumin Concentration on Solubility of Tobacco Mosaic Virus under Various Conditions*

pH of buffer	pH of albumin and virus in buffer	Ionic strength	Serum albumin concentration <i>per cent</i>	Appearance immediately	Appearance after 24 hrs.
2.41	2.63	0.02	1	Clear	Slight ppt.
2.41	2.59	0.02	2	Turbid	Moderate ppt.
2.41	2.58	0.02	3	"	Full ppt.
2.41	2.58	0.02	4	"	" "
4.42		0.1	10	Clear	Clear
4.42		0.1	15	"	"
4.42		0.1	25	Slightly turbid	Slightly turbid
4.42		0.1	35	Turbid	Clear
5.21		0.1	1	Clear	"
5.21		0.1	2	"	"
5.21		0.1	3	"	Turbid
5.21		0.1	4	Slightly turbid	Slight ppt.
5.81		0.1	1	Clear	Clear
5.81		0.1	2	"	Turbid
5.81		0.1	3	Turbid	Ppt.
5.81		0.1	4	"	"

stant, but serum albumin concentration and ionic strength were both varied in a third group of experiments. The virus concentration was 0.3 per cent. Sufficient acetate buffer at pH 5.2 was added to each system to make the ionic strength 0.02. Sodium chloride was added in varying amounts to increase the ionic strength in each of the systems. Serum albumin concentrations of 1, 2, 3, and 4 per cent were used. The results are presented in Table III. It was found that the electrolyte concentration just sufficient to cause essentially complete precipitation in 24 hours varied with the serum albumin concentration. When the serum albumin concentration was multiplied by the critical ionic strength, an essentially

constant value was obtained for the lower albumin concentrations. This indicated that, as a first approximation, the solubility of tobacco mosaic virus in serum albumin was approximately proportional to the product of the ionic strength and the serum albumin concentration.

An experiment was carried out at pH 4.4 to determine whether or not electrolytes affected the solubility of tobacco mosaic virus in serum albumin in the region of opposite charge. The virus was dialyzed until its electrolyte concentration was very low. Then a solution containing approximately 3 mg. of virus and 30 mg. of serum albumin in a total of 1 gm. was

TABLE III

*Effect at Various Serum Albumin Concentrations of Varying Ionic Strength When pH and Virus Concentrations Are Held Constant*

Serum albumin concentration	Molality of NaCl	Ionic strength	Appearance immediately	Appearance after 24 hrs.
<i>per cent</i>				
1	0.18	0.20	Clear	Very slight ppt.
1	0.20	0.22	"	Slight ppt.
1	0.23	0.25	"	Ppt.
1	0.28	0.30	"	"
2	0.080	0.100	"	Clear
2	0.088	0.108	"	Very slight ppt.
2	0.098	0.118	"	Slight ppt.
2	0.110	0.130	"	Ppt.
3	0.046	0.066	"	Slight ppt.
3	0.050	0.070	"	Ppt.
3	0.054	0.074	"	"
3	0.060	0.080	"	"
4	0.032	0.052	"	"
4	0.036	0.056	"	"

prepared by mixing the proteins and titrating with dilute hydrochloric acid until the pH was equal to 4.42. This solution was quite turbid, indicating that, at pH 4.42 in the absence of appreciable amounts of electrolytes, virus is not soluble in a serum albumin solution. Sodium chloride was added to the virus in small amounts until the turbidity disappeared. The ionic strength of the final solution was approximately 0.22. Thus, it appeared that a moderate amount of electrolyte was necessary in order to permit the virus to be soluble in serum albumin at a pH value intermediate between the two isoelectric points. This result is in general agreement with that obtained by Kleczkowski (3).

In order to determine the composition of the precipitate obtained in media alkaline to both isoelectric points, two samples, each containing

approximately 0.02 M acetate buffer at pH 5.2, 3.5 per cent NaCl, 0.5 per cent serum albumin, and 1.0 per cent tobacco mosaic virus, were prepared. In order to insure accuracy, all components were weighed. A precipitate was formed in each, and the supernatant fluid was optically clear in each. Since tobacco mosaic virus solutions are opalescent, this indicates that most of the virus precipitated. Infectivity analyses were carried out by the half leaf method with 64 *Nicotiana glutinosa* leaves per test on the redissolved precipitates and the supernatant liquids. The precipitates were first dissolved in sufficient liquid to make the volumes the same as the supernatant fluids. It was found in both of the duplicate experiments that the average number of lesions per half leaf produced by the reconstituted precipitate at a dilution of  $10^{-4}$  was almost identical with the average number produced by the supernatant fluid at a dilution of  $10^{-2}$ . This shows that about 1 per cent of the infectivity remained in the supernatant fluid and about 99 per cent was found in the precipitate. Eight nitrogen analyses were made on the supernatant fluids of the two samples. Recoveries of 93, 90, 98, 100, 101, 102, 98, and 104 per cent of the serum albumin nitrogen were obtained. The average of the recoveries was 98 per cent. These results show that the precipitates formed in a medium alkaline to both isoelectric points consisted largely if not entirely of tobacco mosaic virus and contained little if any serum albumin. Kleczkowski (3) found that precipitates obtained with tobacco mosaic virus interacting with various proteins of opposite charge contained both virus and the oppositely charged proteins.

The virus separated from serum albumin solutions of like charge in a state highly suggestive of the mesomorphic fibers described by Best (6). When ammonium sulfate was added to the fibers, they were seen to shrink laterally and break up into small segments indistinguishable from the crystals originally described by Stanley (7). The mesomorphic fibers of Best behaved in a similar manner (6).

An electrophoresis experiment was carried out on a solution containing 3 per cent serum albumin and 0.3 per cent virus at pH 5.1 and ionic strength 0.02. At the low ionic strength, the virus was still in solution. Two boundary diagrams were obtained, one of greater area, which moved at a very slow rate, and another of approximately 10 per cent of that area, which moved more rapidly. The latter was probably virus. The indication is, at least as a first approximation, that the virus and the serum albumin migrate independently in an electric field when the composition of the solvent is such as to allow the virus to remain soluble. The sedimentation experiments of Schachman and Lauffer<sup>2</sup> indicate that serum albumin and

<sup>2</sup> Schachman, H. K., and Lauffer, M. A., in press.

the virus are capable of independent sedimentation at a pH value of about 4.7.

#### DISCUSSION

The results of the current investigation lead to the conclusion that, when serum albumin is added, tobacco mosaic virus can be separated from solution in the form of mesomorphic fibers similar to those observed by Best, if the virus and the albumin particles possess the same type of net electric charge. These mesomorphic fibers have been shown by Best to be closely related to the crystals obtained by Stanley by precipitation of tobacco mosaic virus by ammonium sulfate.

In some ways this precipitation resembles the crystallization of tobacco mosaic and other viruses by means of high molecular weight hydrophilic colloids. Cohen (8) observed that heparin, hyaluronic acid and related compounds, starch, and even gelatin were capable of causing viruses to crystallize. His studies were carried out at pH values alkaline to the isoelectric point of tobacco mosaic virus, and all of the hydrophilic colloids used were negatively charged particles.

In the present study, it was shown, first, that tobacco mosaic virus can be crystallized from serum albumin solutions when the charge on the albumin is the same as the charge on the virus particle, second, that the amount of serum albumin needed to precipitate the virus under such circumstances is, as a first approximation, inversely proportional to the electrolyte concentration, and third, that the amount of serum albumin needed to precipitate the virus is, at least at pH 5.2 and 5.8, less when the net charges on both virus and serum albumin are greater. There are forces of repulsion between serum albumin particles and virus particles when they have the same electric charge. The serum albumin apparently has greater chemical affinity for water than the virus has. If the repulsion of the serum albumin particles for a virus particle is greater than the repulsion of other virus particles for the same virus particle, the virus should go out of solution and precipitate, possibly in crystalline form.

When the virus and the serum albumin are of opposite charge, the situation is somewhat different. In this case, there is an attraction between the serum albumin and the virus particles. When the ionic strength is low, this attractive force is sufficiently great to cause a precipitate to be formed. The analyses of Kleczkowski show that such precipitates contain both the virus and the oppositely charged protein. When the ionic strength is increased, the attractive force is decreased, or the salt linkage is weakened, and the virus and the oppositely charged protein dissolve.

## SUMMARY

1. Tobacco mosaic virus can be separated from solution in the form of mesomorphic fibers by the addition of purified serum albumin, when the pH of the medium is such that both have negative charges.

2. The amount of serum albumin required to crystallize the virus depends upon the ionic strength. At pH 5.2, the product of the ionic strength and the serum albumin concentration required just to precipitate the virus is approximately a constant.

3. At constant ionic strength, the amount of serum albumin required to precipitate the virus is greater at pH 5.2 than at pH 5.8.

4. The mesomorphic precipitates are composed, largely, if not entirely, of virus.

5. When the pH of the medium is between the isoelectric points of the virus protein and the albumin, the virus does not precipitate at high values of ionic strength but does precipitate when the ionic strength is considerably less than 0.1.

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# LIPOTROPIC ACTIVITY AND TOXICITY OF METHOXININE (OXYMETHIONINE)

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Methoxinine (DL-2-amino-4-methoxybutanoic acid) has been synthesized by Roblin and coworkers (1), who studied its action on *Escherichia coli* and *Staphylococcus aureus*. The growth-inhibitory effect of this compound was prevented by L-methionine but not by the D isomer. 1 mole of DL-methionine reversed the antibacterial action of 500 to 1000 moles of DL-methoxinine. In combination with sulfonamides a synergistic bacteriostatic effect was produced by methoxinine.

Methoxinine was of interest to this laboratory as a tool in an investigation of the protective action afforded by methionine against the hepatotoxic effects of certain chemicals in protein-deficient states. Provided certain conditions were fulfilled, it might be possible by the use of the oxygen analogue to distinguish between any effect of methionine due to its sparing or replenishment of sulfhydryl enzyme systems (2) and such effect as might result solely from its rôle as a lipotropic factor (3). Before such a demonstration might be made, however, it was first necessary to establish that methoxinine does possess lipotropic activity, in addition to being devoid of toxic manifestations that might otherwise complicate the experiment. The work described in the present report shows that the lipotropic activity does indeed exist, but that methoxinine exhibits an order of toxicity that would probably preclude its use in the connection indicated.

## EXPERIMENTAL

DL-Methoxinine was synthesized<sup>1</sup> by a modification of the procedure of Roblin *et al.* (1). The final product melted in a range of 249–251° (corrected) with decomposition and effervescence. The rate of heating was about 2° per minute above 230°. Elementary analysis gave the following results.

$C_6H_{11}O_3N$ . Calculated.	C 45.10, H 8.33, N 10.52
Found.	" 44.46, " 8.06, " 10.39

The experimental animals were Rockland rats of the Sherman strain, weighing between 130 and 180 gm. Equal numbers of both males and fe-

<sup>1</sup> The authors are indebted to Mr. Joseph Fugger, Department of Chemistry, University of Pittsburgh, for the synthesis of the DL-methoxinine, which was performed under the direction of Dr. Klaus Hofmann of that Department.

males were used. In Series I four groups of ten rats each were fed a lipogenic diet for a period of 21 days, and the supplements of methoxinine or DL-methionine<sup>2</sup> (or both) were given in distilled water solution by stomach tube. In Series II, the amino acids were incorporated into the basal diet to the extent of 0.5 per cent of the latter.

The basal diet consisted of lard 40, casein 5, sucrose 46, salts (4) 4, and cellulose 5. Each animal received daily a tablet supplying approximately 25  $\gamma$  of thiamine hydrochloride, 100  $\gamma$  of calcium pantothenate, 25  $\gamma$  of pyridoxine hydrochloride, and 25  $\gamma$  of riboflavin. In addition, 3 drops of a mixture of 50 ml. of Natola<sup>3</sup> and 1 gm. of  $\alpha$ -tocopherol were given weekly.

In Series I, Group I served as controls, and the others were given the following supplements daily by stomach tube in about 2 ml. of distilled water solution: Group II, 50 mg. of methoxinine; Group III, 50 mg. of methionine; and Group IV, 50 mg. of methoxinine plus 50 mg. of methionine. The last group was included for the purpose of observing any possible antagonistic effect between the two compounds. The experiments of Series II became necessary when it was found that the animals in Group II above did not survive the 21 day experimental period. In Series II, therefore, the amino acids were fed in the diet. Group I of Series II is likewise a control; Group II received a diet containing 0.5 per cent methionine; and Group III, one containing 0.5 per cent methoxinine. Because of the limitation of the quantity of methoxinine available, no group comparable to Group IV of Series I could be included.

All groups were maintained on the experiment for 21 days. Food intake and body weight were recorded weekly. At the end of this period, the animals were exsanguinated by decapitation under light nembutal anesthesia, and the liver was removed, weighed, and analyzed for total fatty acids plus cholesterol by chromate oxidation (5). The kidneys were weighed (combined weight) and portions of each fixed for histological examination.

In the lipide analyses, the livers were ground in a Waring blender with a small quantity of ethyl alcohol, and the pulp transferred quantitatively to a volumetric flask with additional alcohol. Sufficient ethyl alcohol and ethyl ether were then added to fill the flask to the mark with a 3:1 proportion of the two solvents, respectively. The flasks were selected to give a minimum volume of 20 ml. of alcohol-ether mixture to 1 gm. of wet weight of liver tissue, and the tissue pulp was allowed to remain in contact with the extracting fluid, with occasional shaking, for at least several hours be-

<sup>2</sup> From General Biochemicals, Inc., Chagrin Falls, Ohio.

<sup>3</sup> A Parke, Davis and Company preparation containing 55,000 U. S. P. units of vitamin A per gm. and 11,000 U. S. P. units of vitamin D per gm.

fore aliquots of the latter were removed for saponification of the contained lipide. No heating was involved in the extraction procedure. The equivalence, 1 mg. of fatty acid plus cholesterol equals 3.7 ml. of 0.1 N dichromate, which is employed in blood analysis, was arbitrarily taken for use in this work. The total fatty acids plus cholesterol are expressed as percentage of wet weight of the liver.

TABLE I

*Influence of Methionine and Methoxinine Supplements, by Stomach Tube, on Rats Receiving Lipogenic Diet (Series I)*

Group I animals were the controls, Group III received 50 mg. of methionine daily, and Group IV received 50 mg. of methionine and 50 mg. of methoxinine daily.

	Group I	Group III	Group IV
Food intake, gm. per rat per day.....	4.16	4.11	4.24
Weight loss, gm.....	16.60	13.20	34.40*
Liver weight, % of body weight.....	6.10	6.33	5.13*
Kidney " % of body weight.....	0.87	0.92	1.08*
Liver lipide, % of wet organ weight.....	19.90	14.90*	7.02†

\* Deviation from Group I statistically significant.

† Deviation from Groups I and III statistically significant.

TABLE II

*Effect of Incorporating 0.5 Per Cent Methionine and 0.5 Per Cent Methoxinine, Respectively, in Lipogenic Diet (Series II)*

Group I animals were the controls, Group II received the methionine-supplemented diet, and Group III the methoxinine-supplemented diet.

	Group I	Group II	Group III
Food intake, gm. per rat per day.....	6.22	5.76	4.38
Weight loss, gm.....	24.10	26.50	52.30*
Liver weight, % of body weight.....	6.28	4.77*	5.80
Kidney " % of body weight.....	0.82	0.87	1.03*
Liver lipide, % of wet organ weight.....	18.10	5.60*	8.70*

\* Deviation from Group I statistically significant. The liver lipide values of Groups II and III are not significantly different.

### Results

Table I presents the means of the results obtained in Series I in which the amino acid supplements were given in fixed quantity by stomach tube. All the animals in Group II of this series died between the 12th and 20th days of the experiment, and are therefore excluded from any comparison. Table II sets forth similar data for the animals in Series II, in which the



basal diet was supplemented to the extent of 0.5 per cent methionine and 0.5 per cent methoxinine, respectively.

#### DISCUSSION

All groups of animals in both series lost weight during the experimental period, and both the weight loss and food intake were unaffected by supplements of methionine. The addition of 0.5 per cent methoxinine to the basal diet did not alter food consumption, but it markedly accelerated the loss in weight. On the basis of the mean food intake of the group, a level of 0.5 per cent methoxinine in the diet represents an intake of 22 mg. per rat per day as compared with the 50 mg. per rat per day that proved lethal to the animals in Group II of Series I under similar conditions. While no attempt has been made to compare the initial food consumption of this group with that of the others, it may be stated that the appetite of the animals (Group II) was severely depressed. The combination of a nearly equimolar amount of methionine with the methoxinine (Group IV, Series I) brought the food intake up on a par with that of the other groups and permitted survival for the experimental period, but did not accomplish a reversal of the weight loss.

A direct evaluation of the lipotropic activity of methoxinine is not possible from the data in Series I because all the animals in Group II died before completion of the experiment. But the fact that the combination of methoxinine and methionine, as used in Group IV of this series, brought about a significantly greater reduction in liver lipide than the methionine alone is indicative of a lipotropic effect of the oxygen analogue. Direct evidence is afforded in Series II, in which the addition of 0.5 per cent methoxinine to the basal diet produced an effect on liver lipide equal to that of methionine. In both series the food intake of each group was statistically identical with that of the others, so that variation in food consumption is not a factor in the results obtained.

That methoxinine possesses considerable toxicity under the conditions of these experiments is manifest in the complete mortality in 12 to 20 days of the group receiving 50 mg. per rat per day and the extreme weight depletion of the animals ingesting it as 0.5 per cent of the diet. The mechanism of such a toxic effect is not apparent from this work. It may be pointed out, however, that, if this effect represents an interference with methionine metabolism, it is clearly not the transmethylation function of the latter that is blocked. Moreover, the fact that the toxicity is only slightly counteracted by the simultaneous administration of a practically equimolar quantity of DL-methionine leads one to believe that some factors other than this specific metabolite antagonism are operative.

The pathology observed microscopically in sections of the liver correlated reasonably well with the amount of fat found by chemical analysis.

It is worthy of note that the analytical results revealed a greater lipide content of the liver when methionine was given by stomach tube (50 mg. per rat per day) than when it was added to the diet, even though the dietary supplement calculated on the basis of the food intake was only 29 mg. per rat per day. The microscopic sections showed, correspondingly, a more extreme fatty infiltration in the former instance. The sections of kidney were particularly interesting in that those from the animals that succumbed to the 50 mg. per day dose of methoxinine exhibited varying degrees of tubular damage, frequently with glomerular involvement. Some tubular, and occasionally glomerular, changes were found in many of the animals, irrespective of grouping, but the changes in Group II of Series I were more extensive and severe than in any other. This condition seems to have been ameliorated to some extent by the simultaneous administration of methionine as judged from the pathology in Group IV of Series I.

#### SUMMARY

1. With rats fed a diet containing 5 per cent casein and 40 per cent lard, a supplement of 50 mg. of methionine plus 50 mg. of methoxinine per rat per day effected a significantly greater reduction in the lipide content of the liver than did 50 mg. of methionine alone.

2. Addition of either 0.5 per cent methoxinine or 0.5 per cent methionine to the basal diet lowered the lipide content of the liver to the same degree.

3. The administration of 50 mg. per rat per day of methoxinine to animals fed the basal diet produced complete mortality of the group in 12 to 20 days, with significant kidney pathology evident microscopically. Addition of 50 mg. of methionine to this dose raised the level of food consumption, permitted survival over the experimental period, and reduced the severity of kidney damage, but did not prevent a significantly greater depletion of body weight as compared to the controls or to the group receiving methionine alone. This accelerated rate of weight loss was also observed in those animals fed the basal diet containing 0.5 per cent methoxinine (22 mg. per rat per day), although no mortalities occurred in this group over the period indicated.

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## STUDIES OF PYRIDOXINE DISPLACEMENT

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Relatively little work has been done with pyridoxine displacers. Ott (1) found 2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridoxine (desoxypyridoxine) to be a very potent inhibitor of pyridoxine in the metabolism of the chick. 2 molecules of inhibitor were sufficient to offset the vitamin activity of 1 molecule of pyridoxine. In the course of an investigation of antimalarials, McCasland *et al.* (2) synthesized 2-methyl-4-hydroxy-6-hydroxymethylpyrimidine and a number of closely related compounds which are analogues of pyridoxine. No statement was made of their activity. The second paper by this group (3), reports the synthesis of 2,6-di-(hydroxymethyl)-4-hydroxy-5-methylpyrimidine hydrochloride which is the pyrimidine analogue of pyridoxine. Tests for anti-pyridoxine activity of this compound showed it to be inactive. Ott<sup>1</sup> found 2-methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine (methoxypyridoxine) to be nearly as effective a pyridoxine displacer in the chick as was desoxypyridoxine. Mushett *et al.* (4) have reported on the pathologic effects produced by these two analogues of pyridoxine. Atrophy of the lymphoid tissues seemed to characterize the histopathological picture. The effectiveness of desoxypyridoxine in the rat has been reported (5). The Merck group (6) has studied the effect of pyridoxine-displacing agents on the metabolism of tryptophan. From this study, it was concluded that desoxypyridoxine interfered with some phase of tryptophan metabolism. Recently, Beiler and Martin (7) found desoxypyridoxine to be ineffective as an inhibitor of the action of tyrosine decarboxylase. Phosphorylated desoxypyridoxine, on the other hand, displaces pyridoxal phosphate in the tyrosine decarboxylase system.

### EXPERIMENTAL

Several analogues of pyridoxine were prepared and tested with desoxypyridoxine as the standard. These chemicals included 2-acetoxy-3,5-diacetoxymethyltoluene, 2-methyl-3-hydroxy-4-dimethylaminomethylpyridine, 2-methyl-3-hydroxy-4-hydroxymethylpyridine, and 2-ethyl-3-amino-4-ethoxymethyl-5-aminomethylpyridine. The compounds were prepared in accordance with the following procedures.

<sup>1</sup> Ott, W. H., personal communication; cited in (4).

*2-Acetoxy-3,5-diacetoxymethyltoluene* was prepared according to the method of Bruson and MacMullen (8). 110 gm. of 40 per cent formaldehyde were added with stirring to a mixture of 54 gm. of *o*-cresol and 150 gm. of 33 per cent dimethylamine over a period of 1 hour. The temperature was kept at 25–30° during the addition and then raised to 90–95°. After 1.5 hours the product was separated, dried, and distilled under reduced pressure. The yield of the pure product, b.p. 115–117° at 0.3 mm. pressure, was 31 gm. of 2-hydroxy-3,5-bis(dimethylaminomethyl)-toluene.

A mixture of 30 gm. of 2-hydroxy-3,5-bis(dimethylaminomethyl)toluene and 50 gm. of acetic anhydride was heated at 90–95° for 2 hours and then refluxed for 1.5 hours. Distillation yielded 30 gm. of product boiling at 155–160° at 0.3 mm. This was extracted with 1 N hydrochloric acid and redistilled to give 27 gm. of 2-acetoxy-3,5-diacetoxymethyltoluene boiling at 158–159° at 0.3 mm.;  $n_D^{25}$  1.4997.

Analysis— $C_{18}H_{24}O_6$ . Calculated,  $CH_3CO$ —43.8; found,  $CH_3CO$ —43.0

*2-Methyl-3-hydroxy-4-hydroxymethylpyridine Hydrochloride*—A solution of 23 gm. of 4-diethylaminomethyl-3-hydroxy-2-methylpyridine (9) in 31 gm. of acetic anhydride was heated at 90–95° for 3 hours and then refluxed for 1 hour. The resulting mixture was dissolved in water, neutralized, and extracted with ether. Distillation gave 23 gm. of product boiling at 135–136° at 0.3 mm. The hydrochloride melted at 161–162°.

5 gm. of 4-acetoxymethyl-3-acetoxy-2-methylpyridine were dissolved in 200 cc. of 2 N hydrochloric acid and the solution refluxed for 12 hours. The solvent was evaporated under reduced pressure and the residue recrystallized from ethyl alcohol; m.p. 165–166°.

Analysis— $C_7H_9O_3N \cdot HCl$ . Calculated, N 7.98; found, N 8.16

*2-Methyl-3-hydroxy-4-dimethylaminomethylpyridine*—A mixture of 16.4 gm. of 2-methyl-3-hydroxypyridine (10) and 18 gm. of 33 per cent dimethylamine was dissolved in 40 cc. of water and 13.5 gm. of 40 per cent formaldehyde were added over a period of 1 hour. The mixture was allowed to stand overnight; the clear solution was heated to 90–95°, saturated with sodium chloride, and the oily layer separated. Distillation gave 11 gm. of product boiling at 75–76° at 0.5 mm. The hydrochloride melted at 223–224°.

Analysis— $C_8H_{11}ON_2$ . Calculated, N 16.86; found, N 16.93

*Preparation of 2-ethyl-3-amino-4-ethoxymethyl-5-aminomethylpyridine dihydrochloride* occurred by a series of reactions analogous to those used by Harris and Folkers (11) in the preparation of pyridoxine.

To the stirred suspension of sodium amide was added in 5 minutes a solution of 0.3 mole of ethyl methyl ketone in 100 cc. of ether. After 5 minutes 0.4 mole of ethyl ethoxyacetate in 100 cc. of ether was added and the stirring was continued for 2 hours at reflux temperature. The mixture was poured into 300 cc. of water, neutralized with dilute hydrochloric acid, and extracted with ether. The solvent was distilled from the ether solution, the residue added to a hot filtered solution of 40 gm. of copper acetate in 350 cc. of water, and the mixture allowed to stand for 4 hours. The copper salt of the diketone was filtered, washed with ligroin, and recrystallized from methyl alcohol. The yield of pure product, m.p. 137–138°, was 20 gm. or 33 per cent.

The copper salt was stirred with 300 cc. of 10 per cent sulfuric acid and 200 cc. of ether. The acid layer was again extracted with ether and the combined ether solution dried over sodium sulfate. The solvent was distilled and the residue fractionated *in vacuo* through a 30 cm. Vigien column, b.p. 93–95° at 13 mm. The product was 1-ethoxy-2,4-hexadione.

To 63 gm. of cyanoacetamide, dissolved in 450 cc. of hot ethyl alcohol, 102 gm. of 1-ethoxy-2,4-hexadione and 10 cc. of piperidine were added with shaking. The mixture was allowed to stand overnight, cooled, and filtered. The yield of 2-ethyl-4-ethoxymethyl-5-cyano-6-pyridone, after filtration and crystallization from alcohol, was 115 gm., m.p. 174–175°.

To 10 gm. of 2-ethyl-4-ethoxymethyl-5-cyano-6-pyridone in 30 cc. of acetic anhydride containing a little urea were added, with cooling and stirring, 4.5 cc. of fuming nitric acid. The reaction temperature was kept between 5–10° during the addition. The reaction mixture was kept at this temperature for 10 minutes and then at 25–30° for half an hour. The mixture was poured into ice and the crystalline product filtered after 3 hours. The yield of 2-ethyl-3-nitro-4-ethoxymethyl-5-cyano-6-pyridone, m.p. 125–127°, was 4 gm. Recrystallization raised the melting point to 127–128°.

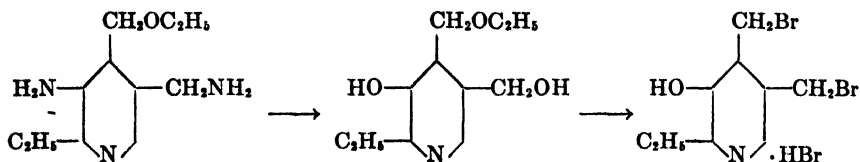
A mixture of 30 gm. of 2-ethyl-3-nitro-4-ethoxymethyl-5-cyano-6-pyridone, 35 gm. of phosphorus pentachloride, and 250 cc. of chlorobenzene was heated at reflux temperature for half an hour and then at such a rate as to distil 150 cc. of the chlorobenzene in 3 to 4 hours. The remaining solvent was removed at 10 to 15 mm. The viscous residue was heated with 80 cc. of 10 per cent ethyl alcohol; the resulting mixture was extracted with ether and dried over sodium sulfate. The ether was distilled and the residue extracted twice with 250 cc. of boiling petroleum ether. This extract was concentrated on a steam bath and then cooled slowly to 10°. The precipitate 2-ethyl-3-nitro-4-ethoxymethyl-5-cyano-6-chloropyridine, after filtration and recrystallization, melted at 52–53°. The yield was 9.5 gm.

A solution of 27 gm. of 2-ethyl-3-nitro-4-ethoxymethyl-5-cyano-6-chloropyridine (m.p. 50–52°) in 175 cc. of alcohol was shaken in the presence of 0.5 gm. of Adams' catalyst with hydrogen at a pressure of 50 pounds. The hydrogenation was stopped after 3 moles of hydrogen had been absorbed and the mixture was allowed to cool. The alcohol was decanted and the product extracted with hot alcohol. Thus 14 gm. of pure 2-ethyl-3-amino-4-ethoxymethyl-5-cyano-6-chloropyridine, m.p. 121–122°, was obtained.

A solution of 3.5 gm. of 2-ethyl-3-amino-4-ethoxymethyl-5-cyano-6-chloropyridine in 140 cc. of glacial acetic acid with 1.2 gm. of sodium acetate, 0.3 gm. of Adams' catalyst, and 5 gm. of 10 per cent palladium charcoal catalyst was shaken with hydrogen at a pressure of 50 pounds until 3 moles had been absorbed (5 hours). After filtering off the catalyst the solvent was evaporated under reduced pressure, and the residue extracted with alcohol and separated from sodium chloride. The alcoholic solution was saturated with dry hydrogen chloride and the dihydrochloride precipitated by addition of acetone. The yield of the pure dihydrochloride of 2-ethyl-3-amino-4-ethoxymethyl-5-aminomethylpyridine, m.p. 214–215°, was 1.2 gm.

Analysis— $C_{11}H_{15}ON_2 \cdot (2HCl)$ . Calculated, N 14.89; found, N 14.93

The structure of 2-ethyl-3-amino-4-ethoxymethyl-5-aminomethylpyridine was established through the following reactions.



A solution of 1 gm. of the dihydrochloride of 2-ethyl-3-amino-4-ethoxymethyl-5-aminomethylpyridine in 20 cc. of water was added simultaneously with a solution of 2 gm. of sodium nitrite to 40 cc. of hot 2.5 N hydrochloric acid. The solution was concentrated to dryness under reduced pressure and the residue washed with acetone. The dihydroxy hydrochloride was extracted from sodium chloride with absolute alcohol and the extract evaporated to dryness.

The crude residue was refluxed with 25 cc. of 48 per cent hydrobromic acid for half an hour, cooled in ice water, and filtered. Two crystallizations from ethyl alcohol gave the pure compound with the same melting point as that given by Harris (12) for 2-ethyl-3-hydroxy-4,5-dibromomethylpyridine hydrobromide.

The method used in testing these agents was to employ the medium of

Atkin *et al.* (13) and the technique of Williams *et al.* (14). The test micro-organism was *Saccharomyces cerevisiae*, G. M.

2-Acetoxy-3,5-diacetoxymethyltoluene and 2-methyl-3-hydroxy-4-di-

TABLE I

*2-Ethyl-3-amino-4-ethoxymethyl-5-aminomethylpyridine As Pyridoxine Displacing Agent*

The results are expressed in readings on the Klett photoelectric colorimeter.

Pyridoxine concentration	Concentrations, $\gamma$ per 10 ml.					
	0	1	10	100	1000	10,000
<i>2-Ethyl-3-amino-4-ethoxymethyl-5-aminomethylpyridine</i>						
$\gamma$ per 10 ml.						
0	102	85	70	63	60	52
0.04	146	103	90	85	68	51
100.04	151	108	102	89	90	63
1,000.04	155	128	123	108	100	95
10,000.04	158	143	123		130	102
Desoxypyridoxine concentrations						
0	110	123	110	80	66	43
0.04	160	123	111	80	74	65
100.04	180	136	123	118	100	92
1,000.04	185	154	150	148	145	125
10,000.04	195	185	185	180	175	135

TABLE II

*Displacement of Pyridoxal by Desoxypyridoxine*

Klett photoelectric colorimeter readings.

Pyridoxal concentration	Desoxypyridoxine concentrations, $\gamma$ per 10 ml.					
	0	1	10	100	1000	10,000
$\gamma$ per 10 ml.						
0	90	120	108	72	53	40
0.0005	162	140	135	70	58	43
100.0005	194	200	160	132	80	72
1000.0005	257	246	245	225	120	101

The ratio is approximately 1:1 in higher concentrations of metabolite and displacer.

methylaminomethylpyridine were inactive. 2-Methyl-3-hydroxy-4-hydroxymethylpyridine was active with an inhibitor-metabolite ratio of 250. 2-Ethyl-3-amino-4-ethoxymethyl-5-aminomethylpyridine was found to be



a more powerful inhibitor of pyridoxine than was desoxypyridoxine. Table I presents an experiment showing comparative results.

The effectiveness of desoxypyridoxine was tested against pyridoxal in order to check the possibility that displacers for one form of vitamin B<sub>6</sub> might not be displacers of the other forms. The results presented in Table II clearly show the effectiveness of desoxypyridoxine in displacing pyridoxal.

To date, only seven compounds have been tested as pyridoxine-displacing agents. It is therefore impossible to generalize concerning the structure of active agents.

#### SUMMARY

Four compounds structurally related to pyridoxine were synthesized and tested as possible displacing agents. Of these, 2-methyl-3-hydroxy-4-hydroxymethylpyridine and 2-ethyl-3-amino-4-ethoxymethyl-5-amino-methylpyridine were found active.

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# ERYTHROCYTE PYROPHOSPHATASE IN HEALTH AND DISEASE

## I. PROPERTIES OF THE ENZYME

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It was previously reported by us (1) that erythrocytes contain an enzyme capable of splitting inorganic pyrophosphate in the presence of magnesium ions. The presence of the enzyme in erythrocytes was mentioned by Jenner and Kay (2) and by Roche (3), but the properties of the enzyme do not seem so far to have been investigated in detail. The pyrophosphatases of some bacteria were studied in some detail by Pett and Wynne (4). Bailey and Webb (5) also have studied the pyrophosphatase of yeast. They found that the enzyme was specifically activated by magnesium ions, and that the activation by magnesium was antagonized by calcium. They also suggested that the yeast pyrophosphatase might be an SH enzyme. The results of a detailed investigation on the erythrocyte pyrophosphatase are outlined in this paper.

## EXPERIMENTAL

**Reagents**—Glass-redistilled water and as far as possible Kahlbaum chemicals were used throughout the investigation.

1. Substrate. 0.01 M sodium pyrophosphate. Pure sodium pyrophosphate was prepared by heating disodium phosphate in a platinum crucible to a red heat until an aqueous solution of it gave no blue color within 10 minutes with Fiske and Subbarow's (6) reagents for orthophosphate. A stock solution of 0.01 M pyrophosphate adjusted by diluting to pH 7.6 with normal HCl was prepared.

2. Activator. 0.1 M magnesium chloride.

3. Buffer. Michaelis' (7) veronal-acetate buffer at pH 7.66.

4. Enzyme. About 10 ml. of venous blood were collected in a 15 ml. graduated centrifuge tube containing the minimum amount of lithium oxalate (or 3 drops of 0.1 M sodium oxalate) and carefully mixed. The blood is centrifuged at 5000 R.P.M. for about 10 minutes. The volumes of the packed red cells and the plasma were noted, and the plasma was pipetted out. 1 ml. of the packed erythrocytes was accurately measured into another centrifuge tube containing normal saline. By careful manipulation of the pipette it is possible to draw out only the red blood cells. The meas-

ured volume of the erythrocytes was washed with 10 volumes of normal saline at the centrifuge. Twice washing with saline was found to be unnecessary. The supernatant saline was pipetted and the erythrocytes were diluted to 10 volumes with water. By shaking, a clear hemolysate was obtained. The enzyme solution thus prepared keeps its potency for more than 2 days, provided it is kept in the ice chest in a glass-stoppered flask. Thereafter the enzyme strength gradually decreases. At room temperature the enzyme rapidly deteriorates, and therefore the hemolysate should be kept in the ice chest as soon as it is prepared, and aliquots taken at the time of assay of the enzyme. Some preparations, and particularly those from guinea pig erythrocytes, retained full activity for more than a week when kept in the ice chest. But generally no preparation which was more than 3 days old was used in the investigation. Attempts to remove the hemoglobin from the enzyme preparation by shaking with chloroform resulted in loss of the enzyme activity.

5. Fiske and Subbarow's reagents for the estimation of orthophosphate (6).

#### *Assay of Erythrocyte Pyrophosphatase*

Into a Pyrex test-tube ( $5 \times \frac{3}{4}$  inches) were measured 3 ml. of the buffer (pH 7.6), 1 ml. of 0.1 M magnesium chloride, 0.5 ml. of the enzyme, and 0.5 ml. of 0.01 M sodium pyrophosphate. The contents were mixed and the tube was placed in a thermostat at 37° for 30 minutes. The reaction was stopped by adding 2.5 ml. of 10 per cent trichloroacetic acid, the tube shaken, and after it had stood for 2 to 3 minutes the contents were filtered. Aliquots of the filtrate were diluted to 5 ml. and the orthophosphate content was estimated by Fiske and Subbarow's method with dilute phosphate standards (5 ml. = 0.08, 0.04, and 0.02 mg. of P). A control experiment (blank) was also set up simultaneously, and the substrate added only after the incubation and just before addition of the trichloroacetic acid. 5 ml. of filtrate were used for the estimation of phosphorus with weaker standards. During the colorimetric estimation the colors must be compared within 5 minutes after the molybdate and the aminonaphtholsulfonic acid reagents are added. Otherwise higher values were obtained, owing to the hydrolysis of the pyrophosphate by the 0.5 N sulfuric acid, and after 15 minutes values more than 10 per cent higher were obtained. The phosphorus equivalent of the 7.5 ml. of the filtrate of the control was subtracted from that of the experiment, and the value multiplied by 20 to give phosphorus, in mg., liberated by the amount of the enzyme present in 1 ml. of the erythrocytes, which is arbitrarily taken as the *unit of pyrophosphatase*.

*Properties of Erythrocyte Pyrophosphatase*

**Magnesium Activation**—At the outset it was found that without added magnesium ions the pyrophosphatase activity of both the erythrocytes and the plasma was negligible, but by the addition of magnesium ions to a concentration of 0.02 M, the erythrocyte enzyme activity increased to more than 100-fold, whereas activity of the plasma enzyme remained practically the same.<sup>1</sup>

The influence of the addition of varying amounts of magnesium chloride to the erythrocyte enzyme buffered at pH 7.66 was studied, and it was found that at very low and at very high magnesium concentrations the enzyme activity decreased, showing thereby an optimum range for full activity. This range lies between 0.02 M and 0.05 M concentration of magnesium. In Fig. 1 *qMg*, a term introduced by Jenner and Kay (2), is plotted against the enzyme activity, expressed as per cent standard assay. (Standard assay refers throughout to the assay under the standard conditions, given above.)

From Fig. 1 it can be seen that rather a high concentration of magnesium is required for optimum activity. The enzyme resembles the erythrocyte alkaline phosphatase (acting on glycerophosphate) in having magnesium as an activator, whereas the erythrocyte acid phosphatase (acting on phenyl phosphate) is reported (8) to be inhibited by magnesium. Various other phosphatases and pyrophosphatases possess an optimum magnesium concentration much less than that of the erythrocyte pyrophosphatase. Yeast pyrophosphatase (5) requires an optimum *qMg* of 2.7, and bacterial pyrophosphatases (4) require a *qMg* of 2.5 for *Clostridium acetobutylicum* and of 4.5 for *Propionibacterium jensenii*; the erythrocyte alkaline phosphatase has a *qMg* of 2.4; the only other enzyme that requires a high magnesium concentration is aminocetylphosphatase (9) from feces. It is reported that this enzyme requires a concentration of 0.08 to 0.1 M magnesium,

<sup>1</sup> A number of experiments were conducted with plasma as well as with the laked red blood cells of the same blood under the same experimental conditions as for the assay, but with 1:5 dilution of plasma instead of 1:10 erythrocytes. In almost all cases there was negligible activation of the plasma pyrophosphatase as shown by the activities with and without added magnesium. The values when magnesium was not added were very nearly equal to the blanks (i.e., substrate added after 30 minutes incubation), and in a few cases they are even less than the blanks, the difference being small and within experimental error. In one case of postarsenical jaundice, with a plasma bilirubin of 5 units and an icterus index of 20, a marked activation of the plasma pyrophosphatase by magnesium was found, the activation being about 70 per cent of that of the erythrocyte pyrophosphatase of the same blood sample. But after 1 week, the same patient's blood was tested, and the plasma was found to have lost its activity.

whereas the aminoethylphosphatase from the kidney requires only 0.005 M magnesium.

In connection with Mg activation, one interesting point observed was that in those experiments in which the pyrophosphate concentration was 0.02 M and above there was turbidity due to the precipitation of magnesium

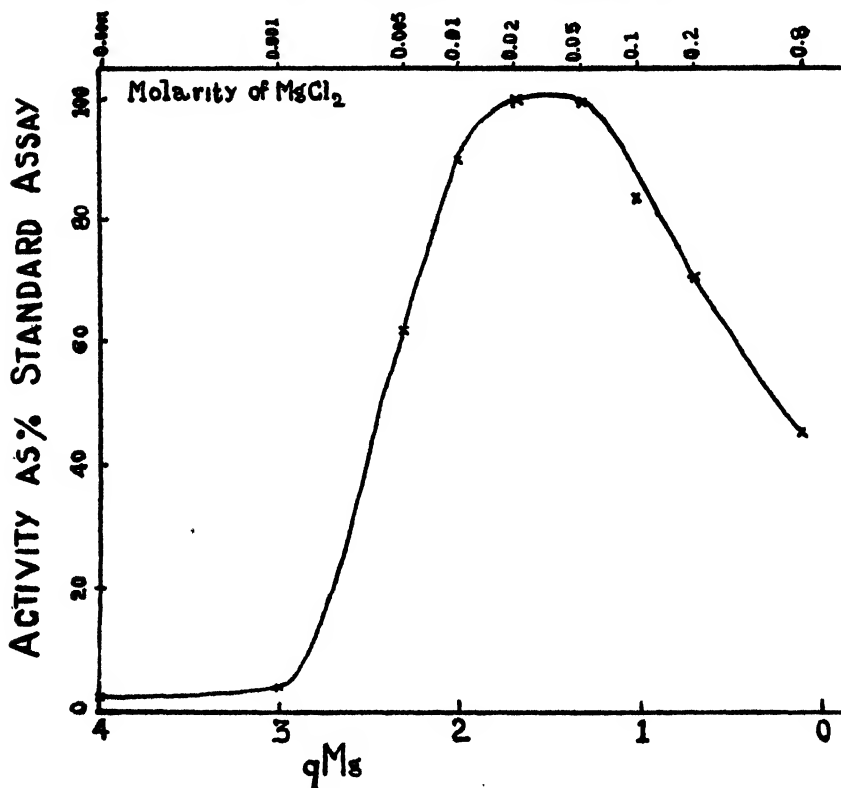


FIG. 1. Magnesium activation curve. 0.5 ml. of enzyme (1:10 red blood cells), 3 ml. of buffer (pH 7.66), 0.5 ml. of 0.01 M sodium pyrophosphate, and 1 ml. of magnesium chloride to give various final concentrations of magnesium. Incubation period, 30 minutes.

pyrophosphate. In Table I are given the results of the effect of varying amounts of the activator and the substrate on the activity of the enzyme.

From the data in Table I it can be seen that the rate of hydrolysis is actually increased, rather than being decreased. Since precipitation caused no depletion of the substrate, or of the activator, it may be inferred that the activator-substrate combination may be essential for the maximum activity of the enzyme. It should also be noted that higher concentrations of magnesium produce an inhibiting effect on the enzyme.

**Protective Action of Magnesium on Enzyme**—It was found that if the enzyme was incubated at 37°, prior to the assay, with buffer alone and *without magnesium*, most of the enzyme activity was lost. But on incubation *with magnesium* the enzyme was protected to an appreciable extent. Table II gives the results of the enzyme activity after incubation *with* and *without* added magnesium for different time periods before the assay.

The results support the view already advanced by Jenner and Kay (2) that the colloid enzyme particle has a high adsorptive capacity for Mg ions, resulting in enzymatic activity. On incubation of the enzyme with buffer without magnesium, the adsorptive capacity of the enzyme surface

TABLE I

*Enzyme Activity with Increased Concentrations of Magnesium and Pyrophosphate, and Effect of Precipitation of Magnesium Pyrophosphate on Activity*

0.5 ml. of enzyme, varying amounts of magnesium and pyrophosphate, and buffer to 5 ml. Incubation period, 30 minutes.

Concentration of magnesium	Concentration of pyrophosphate	Precipitation due to magnesium pyrophosphate	Activity, per cent of standard assay
<i>M</i>	<i>M</i>		
0.01	0.001	Nil	71
0.01	0.002	Slight	90
0.01	0.003	"	99
0.02	0.001	Nil	100
0.02	0.002	Slight	130
0.02	0.003	Marked	138
0.05	0.001	Nil	104
0.05	0.002	Marked	135
0.05	0.003	"	135
0.1	0.001	Nil	87
0.1	0.002	Marked	122
0.1	0.003	"	137

is diminished, owing probably to partial inactivation of the enzyme. From Table II it can be seen that even when magnesium is present there is gradual reduction of the activity.

**pH and Enzyme Activity**—3 ml. of *M*/35 veronal-acetate buffer at various pH values were incubated with 1 ml. of magnesium chloride, 0.5 ml. of the enzyme, and 0.5 ml. of the substrate for 30 minutes, and the phosphate split was estimated. The reaction mixture darkened in the tubes at pH 3.8 and below. In more alkaline reactions, that is above pH 8.9, there was turbidity, due probably to the insolubility of both magnesium hydroxide and magnesium pyrophosphate. Enzyme preparations of different ages were

TABLE II

*Protective Action of Magnesium on Enzyme*

0.5 ml. of enzyme incubated at 37° with 3.9 ml. of buffer, with and without 0.1 ml. of 1 M magnesium for different periods; 0.5 ml. of 0.01 M pyrophosphate added to all, 0.1 ml. of 1 M magnesium to those that do not contain magnesium; standard assay procedure.

Enzyme preparation No.	Incubation period prior to assay	Activity, per cent of standard assay	
		With magnesium	Without magnesium
	<i>min.</i>		
1	15	55	5
2	15	70	12
3	15	66	14
	30	42	
4	15	73	17
	30	38	16
	60	20	7

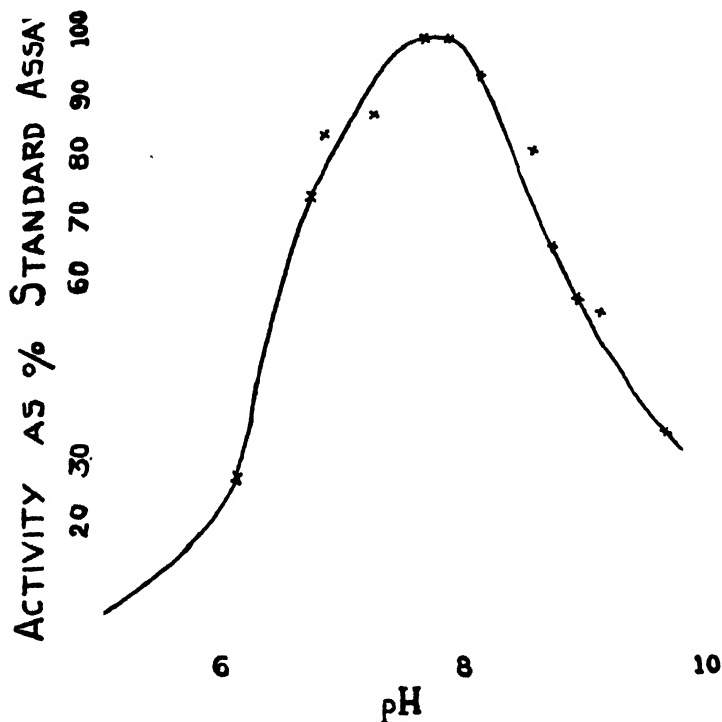


FIG. 2. pH-activity curve. 0.5 ml. of enzyme (1:10 red blood cells), 3 ml. of buffer at various pH values, 1 ml. of 0.1 M magnesium, and 0.5 ml. of 0.01 M pyrophosphate. Incubation period, 30 minutes.

used and the optimum pH range was found to be the same in all cases. Fig. 2 represents the pH-activity curve. The optimum pH range lies between 7.4 and 8.2.

The enzyme differs slightly from yeast pyrophosphatase (5) and bacterial pyrophosphatases (4) in having the optimum pH more towards the alkaline side, as in the case of other mammalian pyrophosphatases (10). Practically negligible activity was shown by the enzyme at both the alkaline pH 8.6 and the acid pH 4.9, which correspond to the optimum for erythrocyte alkaline and acid phosphatases respectively.

*Temperature and Enzyme Activity*—The enzyme activity was determined under standard conditions, but at varying temperatures of incubation. In

TABLE III  
*Enzyme Activity with Temperature*

The enzyme was assayed as under the standard assay except that the temperature was varied.

Temperature	Phosphorus liberated per 1 ml. erythrocytes		
	Enzyme 1	Enzyme 2	Enzyme 3
°C.	mg.	mg.	mg.
5	0.48	0.37	
15	0.75		
27		1.63	
30	2.45		
37	3.71	3.54	3.00
50	3.02		2.50
57		1.31	
67		0.44	

Table III the results of study of three different preparations are given. It can be seen that the optimum temperature lies near the body temperature, and that the optimum range is 30–50°. At 67° the protein was heat-coagulated in the first few minutes, whereas at 57° there was slight coagulation towards the end of the incubation.

*Progress Curve*—The progress curves were studied both with moderate and very strong enzyme preparations. Curve A of Fig. 3 illustrates the results with moderately strong preparations, and Curves B and C with very strong preparations. In the case of Curve A about 65 per cent hydrolysis of the substrate occurs in 3 hours and thereafter the activity becomes negligible. Only about 25 per cent hydrolysis occurs in 30 minutes, whereas with the stronger preparation (Fig. 3, Curve B) about 90 per cent hydrolysis of the substrate occurs in the first 30 minutes and almost 100 per cent in 1 hour. In both cases the hydrolysis curve was linear during the first 30



minutes. This behavior is seen only when the concentration of the substrate is kept low, as is evident from a comparison of Curves B and C, the same active enzyme being used in both cases, but with 6 times more of the substrate in the case of Curve C.

*Enzyme Concentration and Activity*—The enzyme activity of two different preparations at various concentrations and over intervals of 1, 2, and 5 hours

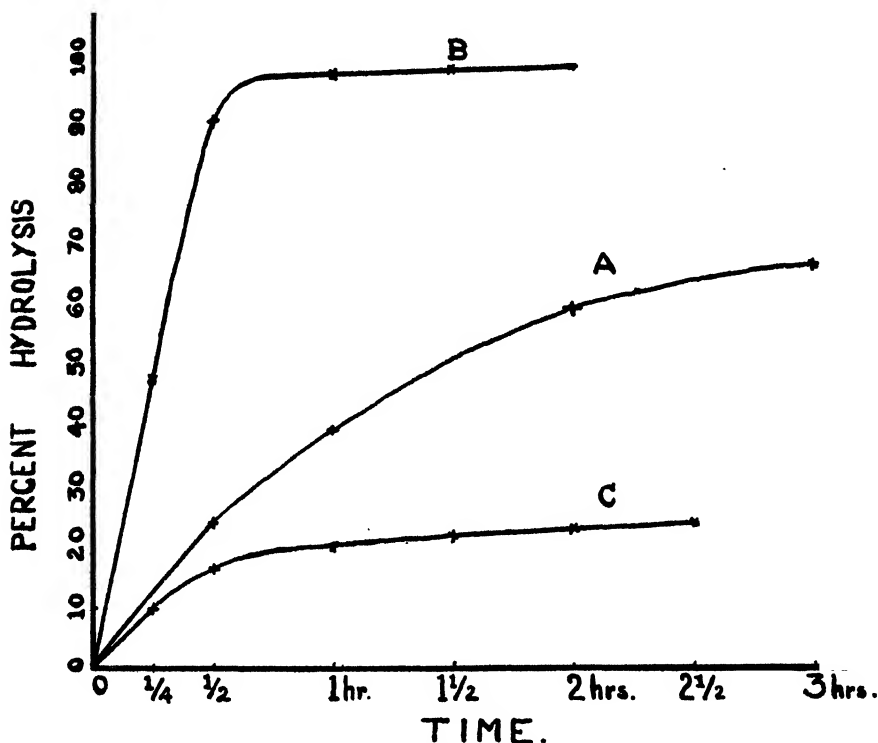


Fig. 3. Progress curve. 0.5 ml. of enzyme (1:10 red blood cells), 3 ml. of buffer (pH 7.66), 1 ml. of 0.1 M magnesium, and pyrophosphate to give a final concentration of 0.001 M in Curves A and B and 0.006 M in Curve C. Total volume, 5 ml. Curves B and C are with the same enzyme preparation; Curve A from another.

was studied, the substrate concentration (0.001 M) and magnesium concentration (0.02 M) in the reaction mixture being constant. With low erythrocyte concentrations, 1:100 (i.e. 1:1000 in the reaction mixture), the activity was rather weak, but in erythrocyte concentrations of 1:20 to 1:5 direct proportionality was found to exist between the enzyme activity and its concentration. Fig. 4 illustrates these results.

*Enzyme Activity and Substrate Effect*—In view of its ability to form metal complexes, pyrophosphate is generally employed in enzyme studies to

detect the presence of any metallic groups that might contribute to the enzyme activity. 0.03 M pyrophosphate is reported to inhibit the respiration of liver tissue in several media (11); inhibition of yeast pyrophosphatase is reported (5) to commence at 0.003 M, giving 50 per cent inhibition in 0.01 M and 100 per cent in 0.03 M pyrophosphate; the purified intestinal

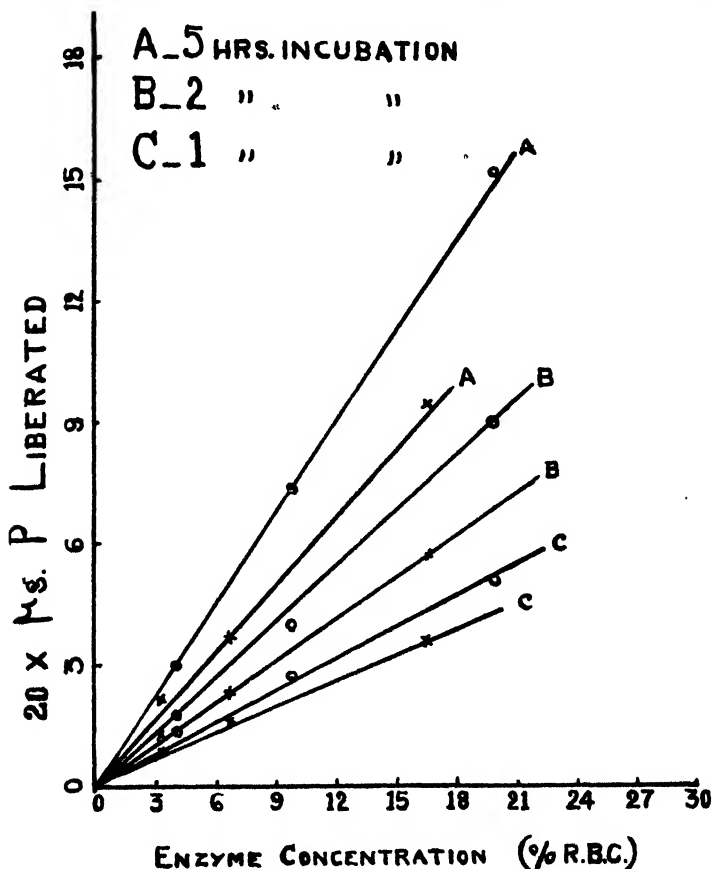


FIG. 4. Enzyme activity with different enzyme concentrations. 0.5 ml. of enzyme of different concentrations, 3 ml. of buffer (pH 7.6), 1 ml. of 0.1 M magnesium, and 0.5 ml. of 0.01 M pyrophosphate. Two different enzyme preparations were used.

alkaline phosphatase also is reported (12) to be inhibited by higher concentrations of pyrophosphate. The pyrophosphatase of red blood cells is also found to be inhibited by 0.01 M pyrophosphate.

The main experimental difficulty encountered in the study of the substrate effect was the interference of the high pyrophosphate concentration on molybdenum blue color production. We had to dilute aliquots of the

test filtrates so as to get a final concentration of less than 0.001 M pyrophosphate before developing the colors.<sup>2</sup>

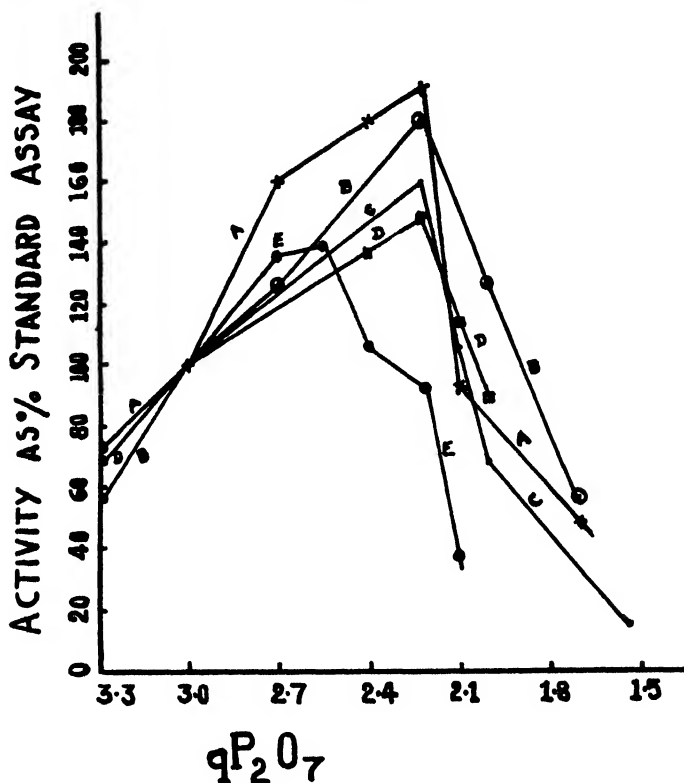


FIG. 5. Enzyme activity and substrate effect. 0.5 ml. of enzyme (1:10 red blood cells), 3 ml. of buffer (pH 7.6), 1 ml. of 0.1 M magnesium, and pyrophosphate to give different final concentrations. Final volume, 5 ml. Incubation period, 30 minutes. Curves A, B, C, and D are for different human erythrocyte enzyme preparations; Curve E, goat erythrocyte enzyme.

Fig. 5 illustrates the results obtained.  $qP_2O_7$  represents the negative logarithm of the molar concentration of the pyrophosphate in the reaction

<sup>2</sup> While pointing out this difficulty, Schmidt and Thannhauser (12) have applied the method of King and Delory (26) for the estimation of phosphorus with "oxin" reagent. They found that with slight modification the method can be employed for the estimation of 2  $\gamma$  or more of phosphorus in a phosphate-pyrophosphate mixture containing not more than 100  $\gamma$  of pyrophosphate phosphorus. But we found by using the modification of Schmidt and Thannhauser that only 4  $\gamma$  or more of phosphorus could be estimated within 10 per cent error at room temperature, 32°, and 2  $\gamma$  or less of phosphorus gave more than 25 per cent higher recovery values. As it is not possible to recover even 1  $\gamma$  of phosphorus in the test filtrates at the high dilution made

mixture. In Fig. 5 it can be seen that with increasing substrate concentration the activity of the enzyme is gradually decreased to negligible values. The splitting of the pyrophosphate progressively increases from 0.0005 to 0.006 M pyrophosphate concentrations, and suddenly the rate falls, as is shown by a steep decline in the curves when the pyrophosphate concentration reaches 0.01 M. The hydrolysis is about 50 per cent of the standard assay at 0.02 M, and at 0.03 M the enzyme activity, though not absent, is negligibly small. Further it can be seen that the maximum hydrolysis occurs in the range of 0.004 to 0.008 M pyrophosphate concentration, and in most cases the range is from 0.006 to 0.008 M. Weaker enzyme preparations (*e.g.* goat erythrocyte enzyme and older preparations of human erythrocyte enzyme) show the inhibitory effect of the substrate at even 0.04 M substrate. It might be that the weaker the enzyme the stronger would be the substrate inhibition at higher concentrations.

*Activators of Erythrocyte Pyrophosphatase*—Of the many substances investigated for activating effect on the enzyme none was found to replace magnesium. *In the presence of magnesium*, bile salt showed definite activation of the enzyme, the extent of activation being dependent upon the age of the enzyme. In the absence of magnesium, the bile salt had practically no activating effect. Similarly very dilute solutions of cyanide (KCN) showed slight activating effect *only in the presence of magnesium*.

The buffered enzyme containing the optimum amount of magnesium was incubated with sodium taurocholate (0.05 per cent salt in the incubation mixture) for 15 minutes, and then the substrate was added and the enzyme assayed. From the results of the enzyme activity with and without bile salt, the percentage activation due to the bile salt was calculated. With very active enzyme preparations, activation due to bile salts was about 20 to 30 per cent and with less potent preparations from 50 to 70 per cent. Older enzyme preparations, which had lost most of their activity, could not be restored to their original activity, but this could be doubled. The bile salt activation is probably due to peptization of the enzyme protein. The cyanide activation, on the other hand, might be due to removal of metal inhibitors.

*Inhibitors of Erythrocyte Pyrophosphatase*—Various substances, which are known to be enzyme inhibitors, were studied to elucidate the nature of the groups that contribute to the erythrocyte pyrophosphatase activity.

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to bring the pyrophosphate concentration to 100  $\gamma$  of phosphorus, we used the method of Fiske and Subbarow. By adding known amounts of orthophosphate to different concentrations of pyrophosphate in water and estimating the phosphate, it was found that the per cent error in the recovery was negligible when the pyrophosphate concentration was 0.001 M or less.

The buffered enzyme containing the optimum amount of the activator (0.02 M Mg) was incubated with varying concentrations of the inhibitors (adjusted to pH 7.6) for 15 minutes, and then the substrate was added and

TABLE IV  
*Inhibition of Magnesium-Activated Enzyme by Different Concentrations of Inhibitor*

Inhibitor		Per cent inhibition				
Ion	Substance used	Final concentration of inhibitor in incubation mixture				
		0.00002 M	0.0002 M	0.002 M	0.01 M	0.02 M
Cu	Copper sulfate	90	100	100	100	100
F	Sodium fluoride	52	95	100	100	100
Zn	Zinc sulfate		96	100	100	100
Ag	Silver nitrate		100	100		
Hg	Mercuric chloride		98	100		
Fe	Ferric alum		0	57	100	
"	Ferrous ammonium sulfate		0*	53	60	
Co	Cobalt sulfate		60	94	100	
Mn	Manganese nitrate			75	100	
Al	Potash alum			0		
Be	Beryllium chloride			31		74
Ba	Barium chloride			60	75	80
Ca	Calcium chloride			80	100	100
CN	Potassium cyanide	0*	0*	0		80
Alloxan				21		100
Iodoacetate	Sodium salt			18		61
Arsenate	" "			66	75	80
Arsenite	" "				21	33
Citrate	" "			10	40	90
Oxalate	" "			0	20	60
Malonate	" "			10	30	70
Succinate	" "			0	0	0
Tartrate	" "			0	0	10
Formaldehyde					90	96
Sulfanilamide				0	0	0
Hydroxylamine				0	0	0
Semicarbazide				0	0	0
Hydrazine				0	0	0

\* Slight activation.

the enzyme assayed. Controls without the inhibitor and blanks with the substrate added just before the trichloroacetic acid were set up simultaneously. From the results the percentage inhibition was calculated. Table IV illustrates the summary of the results obtained.

### *Metallic Inhibitors*

Most of the heavy metallic ions in moderately low concentrations precipitate proteins. Cu, Hg, Ag, and Zn were found to inactivate the enzyme in extremely low concentrations, probably by denaturing the enzyme protein. Zn gave visible protein precipitation in 0.001 M concentration. It also gave almost complete inhibition in 0.0002 M concentration, whereas higher concentrations are reported to be required to inhibit the yeast pyrophosphatase (5). Cobalt and manganese also are fairly inhibitory, cobalt more so than manganese. In this connection it might be mentioned that Pett and Wynne (4, 13) reported appreciable activation of several bacterial pyrophosphatases by zinc in low concentrations, in some cases more than that due to magnesium. Hove *et al.* (14) working with purified alkaline phosphatase showed that appreciable activation of the enzyme could be obtained with minute amounts of zinc (0.04 mM) and also with manganese, and that glycine, alanine, and other  $\alpha$ -amino acids would "coactivate" the zinc activation. King *et al.* (8) also reported activation of erythrocyte acid phosphatase by manganese. But Schmidt and Thannhauser (12) working with purified intestinal alkaline phosphatase could not demonstrate any activation with either zinc or amino acids. We found (data not included in Table IV) that minute quantities of zinc, manganese, glycine, and alanine, produced definite, though very small, activation of erythrocyte pyrophosphatase *only in the presence of magnesium*. Probably the effect would be more marked on the purified enzyme.

Ferric ion was more inhibitory than ferrous ion, but neither produced inhibition at low concentrations in which the other heavy metallic ions showed almost complete inhibition. Ferrous ion was found to cause slight activation when it was employed in very low concentrations (0.0002 M), but only in the presence of magnesium. The slight stimulatory effect might be due to regeneration of some of the groups that might be present in the oxidized form. Aluminum was tried but no inhibition could be obtained. As it forms the hydroxide at the pH employed, it was used only in the form of a suspension. Higher concentrations of aluminum were not tried.

*Effect of Calcium, Barium, and Beryllium*—Calcium-magnesium antagonism is recognized in many of the enzyme reactions in which one of the ions is an activator. Thus the adenosinetriphosphatase of myosin is activated by calcium and inhibited by magnesium, but the same enzyme derived from electrical tissue is activated by magnesium and inhibited by calcium (15); the process of plasma clotting is activated by calcium and inhibited by magnesium (16, 17); the pyrophosphatases of liver and electri-

cal tissue (15) and of yeast (5) are activated by magnesium and inhibited by calcium. With erythrocyte pyrophosphatase also it was found that the magnesium-activated enzyme could be completely inhibited by calcium. Fig. 6 illustrates the results obtained with calcium, barium, and beryllium as inhibitors of the magnesium-activated enzyme. In these experiments,

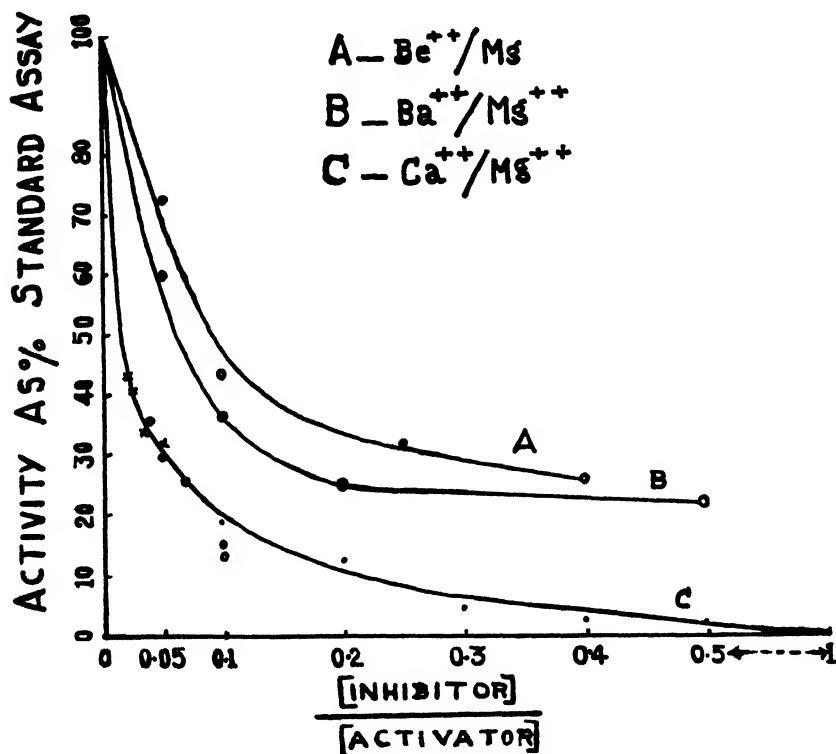


FIG. 6. Antagonism of magnesium with Be, Ba, and Ca. 0.5 ml. of enzyme (1:10 red blood cells), 3 ml. of buffer (pH 7.6), 0.5 ml. of 0.01 M pyrophosphate, and varying concentrations of Mg, Ba, Ca, and Be. Final volume, 5 ml. Incubation period, 30 minutes. Same enzyme preparation in each case. O, magnesium concentration constant (0.02 M), X, calcium concentration constant (0.002 M), ●, higher concentrations of both calcium and magnesium.

the activator and its antagonist were added at the same time, and, without prior incubation, the enzyme was assayed. In estimating phosphorus in those experiments in which higher concentrations of barium were used, we had to add a couple of drops of magnesium sulfate (saturated solution) before precipitating the proteins with trichloroacetic acid, as otherwise the barium would cause turbidity when molybdate in sulfuric acid was added. Beryllium was tried only as a suspension, since it was not possible to get a

clear solution at pH 7.6. From Fig. 6 it can be seen that barium and beryllium also inhibit in the same concentration range as does calcium, but they are less effective, and complete inhibition could not be obtained even at 0.02 M, whereas calcium produces complete inhibition at 0.01 M.

A number of experiments were done with constant magnesium concentration (0.02 M) and varying concentrations of calcium, and with constant calcium concentration (0.002 and 0.004 M) and varying magnesium concentrations, and with uniformly increased concentrations of both to give Ca:Mg = 0.1 to 1.0 M magnesium. The results are summarized in Table V.

From Table V it can be seen that only less than 20 per cent activity remains at Ca:Mg = 0.1. Higher concentrations of calcium with pro-

TABLE V  
*Inhibition of Magnesium-Activated Enzyme with Different Concentrations of Calcium*

Concentration of magnesium	Ca:Mg	Activity, per cent of standard assay	Concentration of calcium	Ca:Mg	Activity, per cent of standard assay
			M		
0.02	0.1	19	0.002	0.05	32
0.02	0.2	12	0.002	0.033	34
0.02	0.3	4	0.002	0.025	41
0.02	0.4	3	0.002	0.02	43
0.02	0.5	2	0.004	0.1	19
0.02	0.75	0.6	0.004	0.066	26
0.02	1.0	0.5	0.004	0.05	30
			0.004	0.04	36
			0.006	0.1	15
			0.008	0.1	15
			0.01	0.1	14

portionate increase in magnesium concentration produce further reduction in activity, and with weaker and older enzyme preparations the calcium effect was found to be still more pronounced. Further, it was found that incubation with both calcium and magnesium for 15 minutes prior to the assay gave in some cases almost complete inhibition with Ca:Mg = 0.1. This point strongly suggests that calcium competes with magnesium for some active centers of the enzyme. It was also found that by using higher concentrations of the substrate the same extent of inhibition, *i.e.* about 75 per cent with 0.006 M substrate and about 85 per cent with 0.001 M substrate, was observed, thereby supporting the point that the calcium inhibition due to substrate depletion must be slight, and that calcium essentially competes with magnesium for some active centers on the enzyme surface.

In view of the activation of the enzyme by sodium taurocholate, we tried



to determine whether bile salt could remove the calcium adsorbed on the enzyme surface and thus prevent inhibition. The results are presented in Table VI, from which it will be noted that bile salt has very little effect. Calcium evidently has a great affinity for some active centers on the enzyme surface. It is also evident from Table VI that calcium inhibition is mainly due to its competition with magnesium for the active centers of the enzyme, and not for the substrate.

Though Be, Mg, Ca, and Ba belong to the same group in the periodic table, the action of calcium seems to be more specific as a divalent activator-competitor. Calcium is physiologically an essential element having a very wide distribution, and the concentration of calcium is always more than

TABLE VI

*Effect of Bile Salt on Calcium Inhibition of Magnesium-Activated Enzyme*

0.5 ml. of enzyme, 1 ml. of 0.1 M magnesium, and substrate and buffer to 5 ml. Two strengths of the substrate were employed to give final concentrations of 0.001 M and 0.004 M pyrophosphate. When calcium or bile salt or both were added, the volume of the buffer was reduced to give a final volume of 5 ml. In the latter cases the enzyme was incubated with the reagents for 15 minutes prior to the addition of the substrate.

		Phosphorus liberated per 1 ml. red blood cells	
		0.001 M substrate	0.004 M substrate
		mg.	
Magnesium-activated enzyme		2.22	3.24
"	+ 0.1 ml. 0.1 M	0.4	0.82
calcium			
Magnesium-activated enzyme + 0.5 ml. 1% sodium taurocholate		4.08	4.45
Magnesium-activated enzyme + 0.1 ml. 0.1 M calcium + 0.5 ml. 1% sodium taurocholate		0.54	1.2

that of the magnesium in any tissue. It is still more interesting to note that the calcium present in blood is almost exclusively distributed in the plasma and not in the erythrocytes. Probably this accounts for the negligible results observed for the plasma pyrophosphatase.

*Effect of Sulfhydryl Inhibitors*

To determine whether the enzyme requires the integrity of SH groups for its activity we tried the effect of substances which influence the SH groups by oxidation or association. From the following results it can be stated that intact SH groups are essential for the erythrocyte pyrophosphatase activity.

*Alloxan*, which acts as an oxidant of SH groups in low concentrations, gave complete inhibition in 0.02 M concentration.

*Copper*, which even in minute amounts is known to react with SH groups and thus inhibit all SH enzymes, gave almost complete inhibition in 0.00002 M concentration.

The inhibitory action of *iodoacetate* is considered to be a relatively slow process (18). But we found that in a concentration of 0.02 M it produced about 60 per cent inhibition.

*Dicarboxylic Acids*—Hopkins *et al.* (18) found that the SH enzyme succinic dehydrogenase was inhibited by malonate, and they regarded the inhibition as due to some effective association of the malonate with some active groups of the enzyme, and considered that these active groups are possibly the SH groups. Moreover, they stated that the most efficient inhibitors of the succinic dehydrogenase are dicarboxylic acids and pyrophosphoric acid, and hence concluded that two acidic groups seem to enter into special relation with the enzyme. We investigated the effect of five di- and tricarboxylic acids, *viz.* oxalic, malonic, succinic, tartaric, and citric acids.<sup>3</sup> Citrate showed more inhibition of the enzyme (see Table IV) than did oxalate or malonate, the latter being fairly inhibitory in 0.02 M concentrations. But the 4-carbon dicarboxylic acids, succinic and tartaric acids, produced practically no inhibition even in 0.02 M concentrations. Probably the special configuration of the substance decides its inhibitory effects on the enzyme.

*Arsenate* and *arsenite* are generally regarded as SH inhibitors. Pett and Wynne (19) have reported that arsenate and arsenite at 0.01 to 0.002 M produce appreciable activation (up to 90 per cent) of the ox erythrocyte enzyme acting on hexose diphosphate at pH 6.5, but the same enzyme acting on glycerophosphate at pH 6.1 showed definite, though slight, inhibition. They regard the acceleration caused by these ions as due to an effect exercised at some stage of the breakdown of the organic portion of the phosphoric ester. We found that both arsenate and arsenite (and also neoarsenobilon) show inhibition in low concentrations, and that arsenate especially destroys more than half of the activity of the enzyme in 0.002 M concentration.

*Fluoride*, *cyanide*, and *pyrophosphate* ions are supposed to form complexes with metal ions, and an enzyme that contains a metallic group as an active group is inhibited by these ions. Fluoride completely inhibits the erythrocyte pyrophosphatase in extremely low concentrations (0.0002 M), whereas

<sup>3</sup> We found that malonate, succinate, and tartrate showed no interference with the color development of molybdenum blue, but that citrate and oxalate showed marked inhibition, and that the interference was abolished when the concentration of the dicarboxylate was reduced to 0.005 M before the colors were developed.

in such low concentrations cyanide showed activation, probably by removing traces of metallic ions such as copper, or by liberating more SH groups. Cyanide and pyrophosphate show inhibition only when their concentrations are about 0.04 M. But this inhibition can be completely abolished by simple dilution to about 10 times. Probably these two inhibitors form some loose complexes with some active groups of the enzyme (perhaps SH or metallic groups), and the complex formation may be dependent on the concentration of the inhibitor. Hopkins *et al.* (18) stated that malonic and other dicarboxylic acids protect succinic dehydrogenase against oxidation by oxidized glutathione, presumably by combining with the SH groups of the enzyme. The combination is so loose as to be easily disrupted by washing. We observed that by dilution it was possible to abolish the inhibitory effects of the dicarboxylates, pyrophosphate, and cyanide.

To find out whether the cyanide and pyrophosphate produce any permanent inactivation of the enzyme by prolonged incubation, the following experiment was set up. The incubation period was 2 hours, as otherwise the enzyme itself shows decrease in activity after longer periods prior to assay. Magnesium was not added during the period of incubation, but only at the time of the assay. As the enzyme concentration in the incubation mixture was 1:20 erythrocytes, the enzyme retained sufficient activity after the 2 hour incubation without magnesium.

Three tubes were set up as follows, with 1:10 erythrocytes as the enzyme and buffer (pH 7.6); Tube A, 3 ml. of enzyme, 1 ml. of buffer, and 2 ml. of water; Tube B, 3 ml. of enzyme, 1 ml. of buffer, and 2 ml. of 0.1 M cyanide; Tube C, 3 ml. of enzyme, 1 ml. of buffer, and 2 ml. of 0.1 M pyrophosphate. All the tubes were incubated for 2 hours at 37°, and then the activity was tested on 1.2 ml. aliquots (corresponding to 0.5 ml. of the enzyme); the details are summarized in Table VII.

From the results it can be seen that by dilution cyanide inhibition is completely abolished (actually more activity is obtained), whereas the pyrophosphate still shows some inhibition. The cyanide-enzyme complex is evidently a loose combination which can be resolved easily by dilution. These two inhibitors may be producing inhibition in higher concentrations by acting differently or on different groups. Since Hopkins *et al.* (18) observed in connection with the inhibitory action of dicarboxylic and pyrophosphoric acids on succinic dehydrogenase that two acidic groups seem to enter into structural relation with the enzyme, our results on pyrophosphate inhibition as well as that of citrate, malonate, and oxalate may be due to such effects.

From the literature we find that the effect of cyanide on various phosphatases is reported to be inhibitory in some cases (2, 12) and without any effect in others (4, 5). Drill *et al.* (20) working with dogs produced evi-

dence that normal serum contains only a very small amount of cyanide-sensitive phosphatase and that the rest is cyanide-insensitive, and that the former is increased abnormally in liver damage.

To ascertain whether the cyanide inactivation is due to interaction with CO groups of the enzyme, the effect of certain keto fixatives was tried.

*Keto Fixatives*—Hydrazine, hydroxylamine, and semicarbazide were used as keto fixatives by Taylor and Gale (21). We tried all three in varying concentrations and found that none gave any inhibition, showing that the enzyme does not require the integrity of CO groups. Higher concentra-

TABLE VII  
*Enzyme Activity after 2 Hours of Incubation with Cyanide  
and with Pyrophosphate*

Solution A, enzyme incubated with water for 2 hours; Solution B, enzyme incubated with cyanide; Solution C, enzyme incubated with pyrophosphate.

	Phosphorus liberated per 1 ml. red blood cells
	mg.
1.2 ml. Solution A, 1 ml. 0.1 M Mg, 0.4 ml. 0.1 M cyanide, 0.5 ml. 0.01 M pyrophosphate, buffer to 5 ml.	2.46
1.2 ml. Solution B, 1 ml. 0.1 M Mg, 0.5 ml. 0.01 M pyrophosphate, buffer to 5 ml.	2.58
1.2 ml. Solution A, 2 ml. 0.1 M Mg, 0.4 ml. 0.1 M cyanide, 1 ml. 0.01 M pyrophosphate, buffer to 10 ml.	2.67
1.2 ml. Solution B, 2 ml. 0.1 M Mg, 1 ml. 0.01 M pyrophosphate, buffer to 10 ml.	3.00
1.2 ml. Solution A, 1 ml. 0.1 M Mg, 0.4 ml. 0.1 M pyrophosphate, buffer to 5 ml.	1.35
1.2 ml. Solution C, 1 ml. 0.1 M Mg, buffer to 5 ml.	0.90
1.2 ml. Solution A, 2 ml. 0.1 M Mg, 0.4 ml. 0.1 M pyrophosphate, buffer to 10 ml.	2.70
1.2 ml. Solution C, 2 ml. 0.1 M Mg, buffer to 10 ml.	1.77

tions of hydrazine could not be used, as it interferes with phosphorus estimation.

*Formaldehyde Inhibition*—Gould (22) studying the action of nitrous acid and formaldehyde on various phosphatases showed that amino groups are essential for the activity of bone, kidney, and intestinal phosphatases. He further observed that crude preparations of the enzyme are more sensitive to low concentrations of formaldehyde than are purer preparations. He suggested that formaldehyde acts on protein associated with the enzyme in the crude preparation. Hove *et al.* (14) found that amino and carboxyl groups in the  $\alpha$  position are coactivators in the activation of dialyzed intestinal phosphatase by zinc. Abul-Fadi and King (23) have recently

reported that formaldehyde inhibits erythrocyte acid phosphatase, while prostatic acid phosphatase is quite unaffected.

From Table IV it can be seen that 0.01 M formaldehyde produces almost complete inhibition of the erythrocyte pyrophosphatase. Both the reaction time and the concentration of the formaldehyde used by us were less than those employed by Gould (0.33 to 0.1 M formaldehyde; reaction time from 6 to 24 hours).

*Miscellaneous*—Sulfanilamide, creatine, and creatinine were tried and found to have no effect on the enzyme. The amount or strength of the pyrophosphatase does not seem to be constant in all persons. A difference in the pyrophosphatase activity of the red cells of different species of animals has also been observed, as in the case of the alkaline phosphatase observed by Jenner and Kay (2). The red cells of goat, sheep, rabbit, dog, guinea pig, and man have been examined, and of these guinea pig corpuscles were found to contain the most active enzyme.

#### DISCUSSION

It has been shown that the red blood cell pyrophosphatase requires magnesium as a coenzyme and that calcium acts as a competitive inhibitor. It was also indicated that the activator-substrate combination produces maximum hydrolysis of the substrate. However, both the substrate and the activator produce inhibition above certain concentrations, the substrate inhibition being much more than that due to the activator.

Bile salt enhances the enzyme activity only in the presence of magnesium, and it has practically no effect in the absence of magnesium. Evidently the function of bile salt is to render more of the enzyme surface accessible to the activator and the substrate.

The results of various SH inhibitors suggest that erythrocyte pyrophosphatase may be an SH enzyme. All reagents reacting with SH produced inhibition. Arsenite, which is supposed to be an efficient SH reagent, produced only slight inhibition. Zittle *et al.* (24) have recently reported that 0.002 M arsenate inhibited the breakdown of di- and monoesters of phosphoric acid (poly- and mononucleotides), whereas arsenite produced no inhibition even in 0.0025 M concentration, and they considered that the usual SH inhibitive effects of arsenate and arsenite were not involved in their experiments. Further they concluded that arsenate inhibition was not due to competition of the inhibitor with the substrate. Barron *et al.* (25) found that the SH enzyme succinic dehydrogenase was inhibited only to a small extent by arsenite when compared to lewisite inhibition. The arsenite inhibition of SH enzymes may not after all be so specific as it was generally assumed.

Though fluoride inhibition indicates the possible presence of a metallic

group, the pyrophosphate and cyanide inhibitions might not have been due to formation of any metal complexes, as the inhibitions in both the latter cases could be completely abolished by dilution.

The formaldehyde inhibition indicates that intact amino groups in the enzyme preparation may be essential. However, the importance of amino groups cannot be assessed unless purified enzyme is tested. The possibility that denaturation of the protein component of the enzyme is the cause of the inhibition cannot be overlooked.

#### SUMMARY

1. Erythrocytes are found to contain a pyrophosphatase which is practically inactive without added magnesium, but shows marked activity in the presence of 0.02 M magnesium chloride.

2. The optimum pH lies in the range of 7.4 to 7.8.

3. The enzyme deteriorates rapidly when kept at 37°, but in the presence of magnesium it seems to retain some of its activity.

4. The enzyme action diminishes with higher concentrations of the activator or the substrate.

5. Magnesium activation of the enzyme is antagonized by calcium or barium.

6. Magnesium activation of the enzyme is enhanced in the presence of bile salt.

7. The enzyme is inactivated by minute amounts of heavy metals and fluoride. Cyanide, arsenic compounds, formaldehyde, and some dicarboxylic acids inhibit the enzyme activity.

8. The inhibition of the enzyme by copper, iodoacetic acid, alloxan, etc., suggests that the erythrocyte pyrophosphatase may be an SH enzyme.

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# A NEW METHOD FOR THE SEPARATION OF ANDROGENS FROM ESTROGENS AND FOR THE PARTITION OF ESTRIOL FROM THE ESTRONE-ESTRADIOL FRACTION

WITH SPECIAL REFERENCE TO THE IDENTIFICATION AND  
QUANTITATIVE MICRODETERMINATION OF ESTROGENS BY  
ULTRAVIOLET ABSORPTION SPECTROPHOTOMETRY\*

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It is recognized generally that a qualitative and quantitative knowledge of the excretion pattern of the urinary estrogens is one index to an understanding of the functional activity of the ovary and adrenal cortex. Obviously, such determinations may be useful also in evaluating the normal and abnormal functions of other physiologically related endocrine glands as well as of organs like the liver and kidneys. The clinical applications of these data are self-evident.

Various attempts have been made to circumvent the notoriously inaccurate values which have been obtained for the urinary estrogens by a variety of bioassay methods and colorimetric techniques (1, 2). The acknowledged shortcomings of these methods have led us to investigate the application of ultraviolet absorption spectrophotometry to the quantitative determination of the urinary estrogens in an attempt to develop an objective physical method for their accurate determination. It is known that the infra-red portion of the spectrum yields more differentially characteristic curves, but those of the ultraviolet range are more readily obtainable, and consequently better adapted to clinical use.

This communication is concerned with studies of the following aspects of the problem: (1) spectrophotometric identification and quantitative micro determination of crystalline estrogens; (2) detection by spectrophotometric assay of gross errors in current methods for extraction and partition of estrogens; (3) studies on the ultraviolet absorption of sub-

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stances comprising the background material; (4) separation of the phenolic estrogens from the so called neutral steroid fraction; (5) separation of urinary estrogens from other urinary phenolic substances by steam distillation; (6) micro-Girard separation of estrone from estradiol; (7) an essentially new method for the extraction and partition of crystalline estrone, estradiol, and estriol, and their quantitative assay by ultraviolet spectrophotometry.

*Spectrophotometric Identification and Quantitative Microdetermination of Crystalline Estrogens*

Ultraviolet absorption spectra of chemically pure crystalline estrone, estradiol, and estriol were determined with the Beckman quartz spectrophotometer. The region from 226 to 300  $m\mu$  was investigated, and curves were constructed from points determined at intervals of 2  $m\mu$ . The corrected constant melting point of each of these compounds was determined in order to establish the purity of the compounds. These values were found to be 258° for estrone, 273–274° for estriol, and 176.7° for  $\alpha$ -estradiol.

*Experimental Procedures*—4 or 5 ml. of U. S. P. 95 per cent ethyl alcohol were used throughout these experiments as a solvent for the residues of the substances that were subjected to ultraviolet spectrophotometry. This solvent has the advantage of being transparent from the visual range to 200  $m\mu$ . The alcohol was not redistilled, because its use as the blank for the setting of the spectrophotometer compensated adequately for the insignificant amount of absorption due to its impurities. All of the experiments currently reported were controlled in this fashion.

25 mg. of each of the three estrogens were dissolved in 95 per cent ethyl alcohol and made up to volume in a 25 ml. volumetric flask. Aliquot portions of each standard were diluted quantitatively subsequently to give concentrations of 125, 100, 75, 50, 25, and 12.5  $\gamma$  per ml. These dilutions were used in the construction of the individual calibration curves.

*Results*—The ultraviolet absorption curves of estrone, estriol, and estradiol were found to be very similar. Our observations are in essential agreement with those in the literature (3–8) in so far as the *general* shape of these absorption curves is concerned. The estrone and estriol are characterized by a minimal density at 248  $m\mu$  and maximal densities between 280 and 282  $m\mu$  with a secondary peak at 288  $m\mu$ . Another maximal density is to be found below 230  $m\mu$ . Estradiol shows the same peaks as estrone and estriol, but the lowest extinction occurs at 252  $m\mu$ . The secondary peak, which was observed at 288  $m\mu$  in the curves for all three estrogens, is to be found in the absorption curves for estrone published by Hogness *et al.* (3), Mayneord and Roe (4), and Callow (5), although they do not call attention

to this point specifically in their texts. The constancy of the appearance of this secondary peak in the curves for all three estrogens suggests that it may be of significance in connection with the molecular structure of these substances. Between 280 and 288  $m\mu$ , our absorption curve for estrone is in general accord with that of Heard and Hoffman (6), but it differs considerably from the characteristics of their curve below and above these points.

Although the data in the literature indicate that the spectrophotometric curves for estrone, estradiol,\* and estriol are essentially identical, we have

TABLE I  
*Differences in Densities and Molecular Extinction Coefficients  
of Estrogens at 280  $m\mu$*

Estriol		Estrone		Estradiol	
$\gamma$ per ml.	Density	$\gamma$ per ml.	Density	$\gamma$ per ml.	Density
	100 $\gamma$ per ml.		100 $\gamma$ per ml.		100 $\gamma$ per ml.
25	0.716	25	0.760	50	0.754
50	0.664	50	0.760	52	0.769
52	0.664	75	0.774	75	0.717
75	0.713	98	0.793	99	0.707
92	0.714	100	0.758	100	0.689
100	0.682	103	0.779	101	0.724
101	0.710	107	0.769	105	0.734
104	0.734	125	0.740	112	0.734
108	0.708			125	0.736
125	0.690				
Average . .	0.700		0.767		0.729
	2000		2073		1970

\*  $\epsilon = (1/cx) \log_{10} (I_0/I_x)$ , in which  $\epsilon$  = molecular extinction coefficient,  $c$  gm. molecules per liter,  $x$  = width of cell in cm.,  $\log_{10} (I_0/I_x)$  = density.

found a significant difference in the density per unit weight at a given concentration at 280  $m\mu$  (Table I). The average density values for 100  $\gamma$  per ml., as determined by a series of experiments of varying concentrations, are 0.700 for estriol, 0.767 for estrone, and 0.729 for estradiol. These differences are not due solely to differing molecular weights. Apparently, the carbonyl group in Ring D of estrone enhances its density at 280  $m\mu$ , because this group exhibits an absorption maximum in that region. On the other hand, the hydroxyl groups in Ring D of estriol and estradiol show a maximal absorption at 186  $m\mu$ , and therefore do not influence the density values at 280  $m\mu$ . So far as the difference between estriol and estradiol is concerned, one should note that on an absolute weight basis there are a larger number

of estradiol molecules than estriol molecules in a solution of a given concentration. This is in accord with the experimental data, which indicate that the density of the estradiol solution is greater than that of the estriol solution.

We have also determined that the minimal density of the estradiol differs consistently, but not remarkably, from that of the estrone and estriol. Since this difference is relatively small, these three estrogens must be

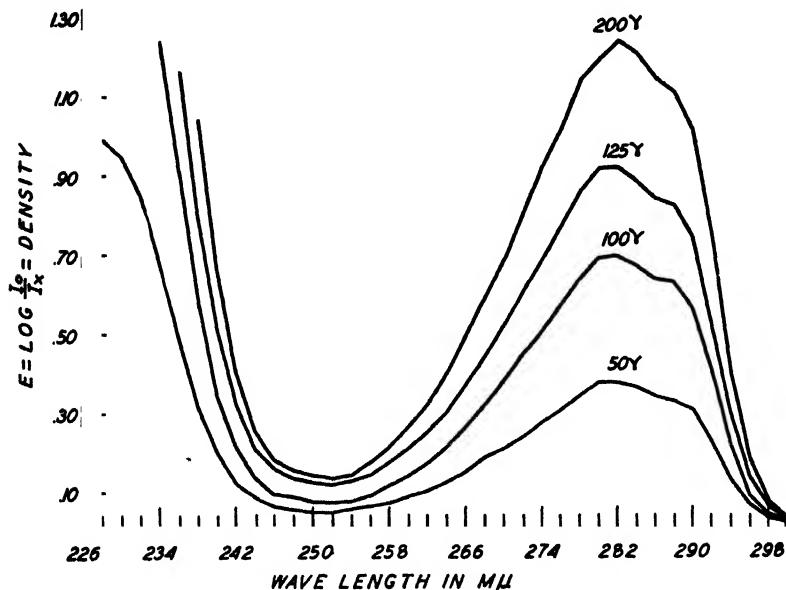


FIG. 1. The effect of concentration upon the ultraviolet absorption of estradiol

separated quantitatively before the spectrophotometric method can be used for their determination.

The effect of concentration of estradiol upon the ultraviolet absorption is shown in Fig. 1. As might be expected from the decreased sensitivity of the instrument above  $E = 1.00$  and below  $E = 0.10$ , the extinction coefficient is proportional to the concentration only between those limits.

The peak at 280  $m\mu$  was selected for the construction of a calibration curve for each of the estrogens (Fig. 2). It is clear from these data that the relation between density and concentration follows Beer's law between  $E = 0.10$  and  $E = 1.00$  at a wave-length of 280  $m\mu$ . The reproducibility of results at a wave-length of 280  $m\mu$  was tested on 10 successive days with each of the three estrogens in dilutions of 100  $\gamma$  per ml. The percentage variation from the mean was found to be negligible (Table II). The data

on the concentration-extinction relationship in Fig. 2 are not in agreement with those of Reynolds and Ginsburg (7). Since they omitted reference

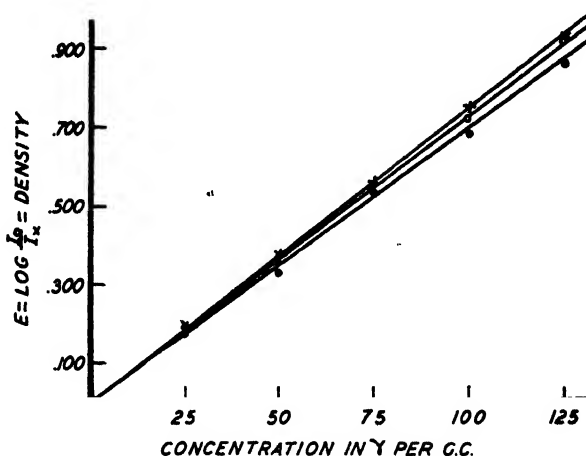


FIG. 2. Calibration curves of estrone, estradiol, and estriol, respectively, in 95 per cent ethyl alcohol at a wave-length of 280  $m\mu$ . X = estrone; O = estradiol; ● = estriol.

TABLE II  
*Reproducibility of Density Values for Estrogens at 280  $m\mu$*

Estrone		Estradiol		Estriol	
Density	Per cent variation from mean	Density	Per cent variation from mean	Density	Per cent variation from mean
0.824	0.2	0.708	0.4	0.700	0.4
0.830	0.5	0.718	1.0	0.695	0.3
0.815	1.1	0.709	0.3	0.690	1.0
0.824	0.2	0.705	0.8	0.696	0.1
0.828	0.2	0.708	0.4	0.700	0.4
0.825	0.1	0.712	0.1	0.700	0.4
0.824	0.2	0.720	1.3	0.700	0.4
0.838	1.4	0.712	0.1	0.700	0.4
0.830	0.5	0.712	0.1	0.695	0.3
0.820	0.7	0.710	0.1		
Mean . . . 0.826		0.711		0.697	

to the melting points of their preparations, there is no way of determining the purity of the hormones employed in their studies.

One may conclude from the calibration curves of Fig. 2 that each of the estrogens may be determined with accuracy only in concentrations above 12 to 15  $\gamma$  per ml. of alcoholic solution. Various studies indicate that the total estrogen excretion in certain phases of the menstrual cycle may be such that the concentration of estrogens in the final alcoholic extracts is well below this amount. This would make it necessary in certain circumstances to extract pooled urine specimens of several days excretion in order to determine their estrogen content. We have applied the technique of lyophilization to urine in order to make it possible to collect and concentrate it in relatively large quantities without running the risk of chemical alteration of its steroid content (9).

*Detection by Spectrophotometric Assay of Gross Errors in Current  
Methods for Extraction and Partition of Estrogens*

*Critique of Currently Accepted Methods for Partition of Estrogens*—A detailed analysis of the literature on data obtained by methods in general use for the partition and assay of urinary estrogens discloses that the quantitative accuracy claimed for them is not in accord with the results on which this contention is based. The foregoing statement may seem startling in view of the wide-spread and relatively unquestioned acceptance of these methods. The facts speak for themselves, however.

Working with aqueous solutions of crystalline estrogens, Cohen and Marrian (10) observed that their method of partition yielded a recovery of 78 to 93 per cent of estriol and 96 to 106 per cent of estrone. Furthermore, from the urines to which crystalline estriol and estrone were added, respectively, they recovered 97 per cent of the estriol and 93 per cent of the estrone. In the light of what we now know of the expected 20 per cent loss of estriol in the  $\text{Na}_2\text{CO}_3$  washings, added to the inevitable large loss of estriol during a 2 to 4 hour hydrolysis at pH 1 to 2, these high recoveries are open to question. As a consequence, one is inclined also to wonder about the quantitative accuracy of their values for the recovery of estrone, particularly since they themselves have acknowledged that in the estrone-estriol partition 20 per cent of the estrone is carried over into the estriol fraction.

Using the Cohen and Marrian technique, except for a recognized improvement in the conditions for hydrolysis, Smith, Smith, and Schiller (11) found that the results obtained in *exploratory partition experiments*, which they singled out for special study, could not be duplicated when estrogens were submitted to the *entire procedure of extraction and partition*. They reported that the three estrogens could be separated quantitatively when submitted to partition alone. However, when carried through the entire procedure, they observed that the recovery of estrone was between 60 and 105 per

cent, that the recovery for estriol varied between 33 and 103 per cent, and that estradiol was recovered in amounts varying from 40 to 99 per cent of the added material.

To explain this discrepancy, Smith, Smith, and Schiller attributed the inconsistent results to an inadequate recovery from NaOH of the separated estrogens, rather than to the Cohen and Marrian technique of partition. Although they claimed uniformly higher recoveries of added estrogens when they modified the technique in accordance with their theory, an analysis of the data suggests that their conclusions were based on insufficient evidence.

In view of the foregoing, it would seem that the data in the literature have not been examined critically enough. The fact that nearly perfect recoveries were obtained under experimental conditions in which substantial losses are known to occur leads us to question the validity either of the methods of assay or the extraction and partition procedures, or both. In this connection, one may consider the data published by three reliable groups of investigators, who checked the Kober colorimetric assay for urinary estrogens against the bioassay method. It is well recognized that the Kober reaction is affected by non-estrogenic urinary substances, and consequently gives overestimates of the actual total estrogen content. The error thus introduced may be one of the factors contributing to the vast discrepancies disclosed by the data in Table III. For example, two samples analyzed by the bioassay method showed 1.100 and 1.150 mg. of estrone, respectively, as compared with 0.672 and 4.140 mg. of estrone determined colorimetrically. Reference to the ratios for the Kober colorimetric-bioassay in Table III shows that these difficulties are the rule rather than the exception, and that they are of a significant order of magnitude. In spite of the fact that perennial attempts have been made to establish the accuracy of one of these methods by checking it against the other, the results in Table III indicate that this cannot be done.

Because of the doubt which has been expressed concerning the quantitative accuracy of the bioassay and the Kober colorimetric techniques, and because we have demonstrated already that the spectrophotometric method can be used for the quantitative assay of crystalline estrogens, it seemed advisable to evaluate the efficiency of the currently accepted partition methods by this physical means.

There are in common use today two types of methods for the separation of estriol from the estrone-estradiol fraction. This partition is accomplished generally by the equilibration of either an alkaline salt or a hydroxide with one of several organic solvents.

Ultraviolet absorption spectrophotometry, as a method of assay in the partition of estrogens, could not be applied satisfactorily to the Cohen and Marrian technique because of the toluene used in the procedure. Toluene

TABLE III

*Illustrations of Inconstant Relations Between Kober Colorimetric and Bioassay Methods*

Period of gestation	Estrone			Estriol		
	Kober	Bioassay	K:B*	Kober	Bioassay	K:B*
Cohen and Marrian (10), mg. per 100 ml. urine						
Sample 3†				0.205	0.243	0.84
" 3†				0.240	0.231	1.04
" 4†	0.033	0.024	1.38	0.182	0.171	1.06
" 4†	0.042	0.031	1.36	0.178	0.178	1.00
" 5†	0.071	0.070	1.01	0.316	0.265	1.19
" 5†				0.306	0.218	1.40
" 5†				0.275	0.224	1.23
" 5†				0.280	0.231	1.21
" 6a†	0.100	0.107	0.94	0.691	0.624	1.11
" 6a†				0.700	0.607	1.15
" 6b†	0.127	0.126	1.01			
" 6b†	0.100	0.115	0.87			
" 7a†				0.680	0.593	1.15
" 7a†				0.705	0.558	1.26
" 7b†	0.100	0.092	1.09			
" 8†	0.127	0.125	1.02	1.070	1.070	1.00
" 8†	0.123	0.121	1.02			
Smith, Smith, and Schiller (11), mg. per 24 hr. sample						
3 mos.	0.840	0.060	14.00	3.960	0.146	27.12
5½ "	0.900	1.300	0.69			
6 "	3.020	2.500	1.21	10.800	10.700	1.01
7 "	2.254	1.350	1.68	13.600	10.800	1.26
8 "	2.368	2.450	0.97	30.710	11.800	2.60
8 "	4.270	5.100	0.84	39.979	24.500	1.63
8 "	0.672	1.100	0.61	38.000	10.600	3.59
8 "	4.140	1.150	3.60	36.400	7.350	4.95
41 days before delivery	1.800	0.800	2.25	14.000	10.700	1.31
22 " " "	3.040	1.500	2.03	11.600	8.000	1.45
15 " " "	5.400	6.600	0.83	46.880	64.000	0.74
9 " " "	3.600	2.500	1.44	42.080	21.300	1.98
6 " " "	2.520	1.335	1.89	34.080	21.200	1.61
Pincus, Wheeler, Young, and Zahl (12), rat units						
Approximately 5th day	606	148	4.09	647	148	4.37
" 5th "	432	252	1.71	1812	840	2.16
4 mos.	1140	675	1.69			
4½ "	1318	450	2.93	1241	685	1.83
5 "	663	330	2.01	998	600	1.66
5½ "				1463	1780	0.82
6 "	1044	550	1.90	1788	2200	0.81
7 "				1502	1800	0.83
7-9 "	891	670	1.33	5800	8900	0.65

\* (Colorimetric data)/(bioassay data). † Period of gestation not stated.

exhibits a significant optical density between 230 and 290  $m\mu$  (13), a range which includes the absorption peak of the estrogens. The present experiments were limited, therefore, to a consideration of those methods in which estriol was separated from the estrone-estradiol fraction by the use of  $\text{Na}_2\text{CO}_3$  and benzene. Consequently, the investigation centered around the Mather principle for the partition of estrogens; namely, the use of  $\text{Na}_2\text{CO}_3$  to remove estriol from the benzene solution. This principle is incorporated in the methods recommended by Pincus (1) and by Bachman and Pettit (14). In view of these considerations, one of the methods selected for study was the Pincus modification of the Mather technique (1). The other method studied spectrophotometrically was that originally recommended by Mather (15). Since neither Mather nor Pincus attempted any purification of the so called "strong" and "weak" phenolic fractions, it seemed feasible to determine whether such a procedure would facilitate the application of the spectrophotometric technique to the assay of estrogens. The Bachman and Pettit method (14) was used for the process of purification after the Mather partition. It was hoped that these steps would prepare extracts relatively free from background material, which is anathema to the spectroscopist.

*Experimental Procedure*—2.569 mg. of estrone, 2.793 mg. of estradiol, and 2.532 mg. of estriol were each dissolved in 25 ml. of 95 per cent ethyl alcohol. 20 ml. of each of these solutions were pooled. Two 25 ml. aliquots of this pool were evaporated to dryness with suction. The residues of these aliquots were subjected to the methods of partition selected for study (Tables IV and V).

*Results*—The results of these experiments, which are to be found in Figs. 3 and 4, are clear cut. The following conclusions concerning the estrone-estradiol fraction may be drawn from these data: (1) The experimental curves (P and M-BP) in Fig. 3 show that the shapes of the curves are characteristic of estrogens, and that the minimal and maximal densities occur at 248 and 280  $m\mu$ . (2) Judging by the relatively high level of the minimal densities of Curves P and M-BP at 248 and 296  $m\mu$ , there is an appreciable amount of background material present. Since it is probable that the maxima at 280  $m\mu$  have been affected similarly, the data at this point cannot be taken at face value. (3) Because of essentially equal amounts of background material, a rough comparison can be made between Curves P and M-BP at 280  $m\mu$ . Judging by the differences between the minimal densities of the experimental (P and M-BP) and the standard (S) curves, it would appear that about three-quarters of the estrone-estradiol mixture was recovered after the Pincus modification, and that approximately one-half of these estrogens was recovered after the Mather-Bachman and Pettit procedure. On the basis of this observation, it occurs to us that



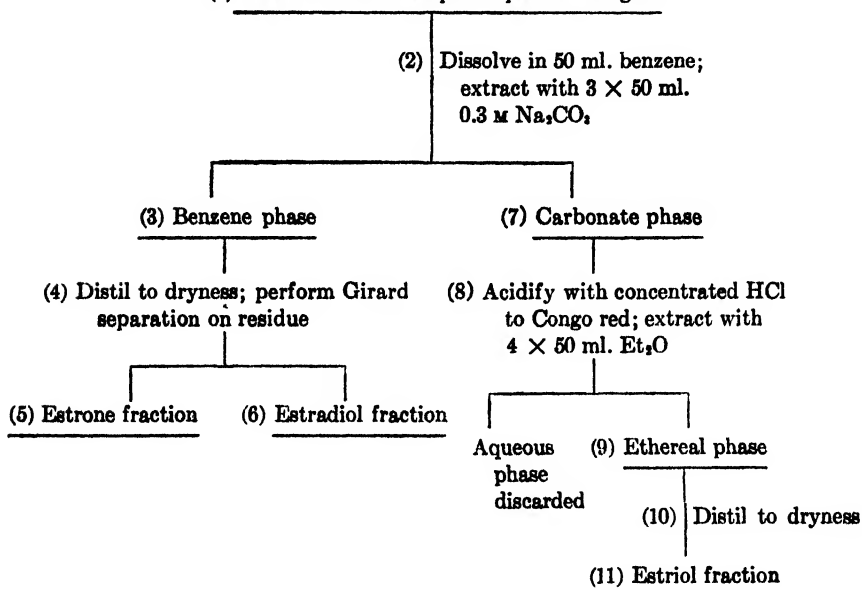
whatever one stands to gain by the manifold attempts to remove urinary impurities by the Bachman and Pettit procedure may be more than over-balanced by the losses sustained in these extensive chemical manipulations.

A similar study was made of the estriol fraction treated by the two methods which have been described. Analysis of the curves in Fig. 4 discloses a marked discrepancy between the density of the absorption peaks of recovered estriol (Curves P and M-BP) and that of the standard solution (Curve S), which represents the quantity of estriol with which each experiment was started. It should be noted in connection with Curves

TABLE IV

*Pincus Modification of Mather Partition*

(1) Residue of 25 ml. aliquot of pooled estrogens



P and M-BP that their configuration is distorted greatly by the background material, inasmuch as the characteristic outline of the estrogen curve is no longer recognizable. Judging from the amount of background material indicated by the readings at 248 and 296  $m\mu$ , the recovery of estriol is practically nil. The latter is not surprising in view of the relatively low solubility of estriol in benzene, a point which was not taken into account either by Mather or Pincus (15, 1). In confirmation of Bachman and Pettit's (14) observation, we have found that the preliminary solution of estriol in alcohol is absolutely essential if estriol is to be taken up by

TABLE V

*Mather Partition with Bachman and Pettit Purification of "Weak" and "Strong" Phenolic Fractions*

- (1) Residue of 25 ml. aliquot of pooled estrogens
- (2) Dissolve in 100 ml. 0.3 M  $\text{Na}_2\text{CO}_3$ ; extract with  $2 \times 50$  ml. benzene
- (3) Carbonate phase
- (4) Wash with 25 ml. benzene; add wash to benzene phase
- (5) Washed carbonate phase
- (6) Extract with  $3 \times 50$  vol. %  $\text{Et}_2\text{O}$
- (7) Ethereal phase
- (8) Wash with 10 ml. 9%  $\text{NaHCO}_3$
- (9) Washed ethereal phase
- (10) Distil to dryness; dissolve residue in  $3 \times 25$  ml. benzene; wash with 10 ml.  $\text{NaHCO}_3$ ; extract with  $3 \times 25$  ml.  $\text{H}_2\text{O}$
- (11) Aqueous phase
- (12) Evaporate *in vacuo*
- (13) Estriol fraction
- (14) Benzene phase
- (15) Wash with 25 ml.  $\text{Na}_2\text{CO}_3$ ; add wash to carbonate phase
- (16) Washed benzene phase
- (17) Extract with  $3 \times 50$  ml. 2 N  $\text{NaOH}$
- (18) Benzene phase
- (19) Wash with  $2 \times 25$  ml.  $\text{H}_2\text{O}$ ; add wash to aqueous phase; discard benzene
- (20) Alkaline phase
- (21) Acidify with concentrated  $\text{HCl}$ ; extract with  $3 \times 50$  ml. benzene
- (22) Benzene phase
- (23) Concentrate to 50 ml.; wash with  $\text{H}_2\text{SO}_4$  (4:5),  $\text{Na}_2\text{CO}_3$ , and  $2 \times 25$  ml.  $\text{H}_2\text{O}$
- (24) Benzene phase
- (25) Distil to dryness; perform Girard separation on the residue
- (26) Estrone fraction
- (27) Estradiol fraction

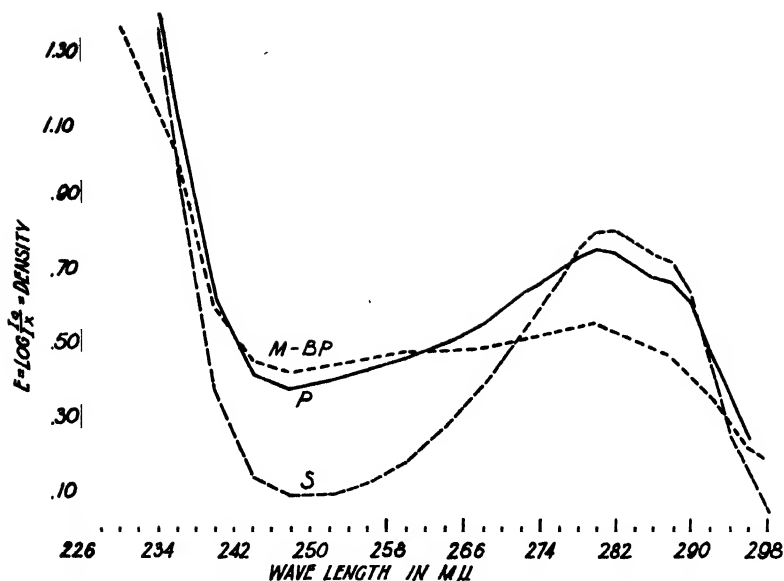


FIG. 3. Comparison of the ultraviolet absorption curve of a standard estrone-estradiol solution (Curve S) with the curves representing the amounts recovered from identical aliquots by the Pincus modification of the Mather partition (Curve P) and by the Mather-Bachman and Pettit partition (Curve M-BP).

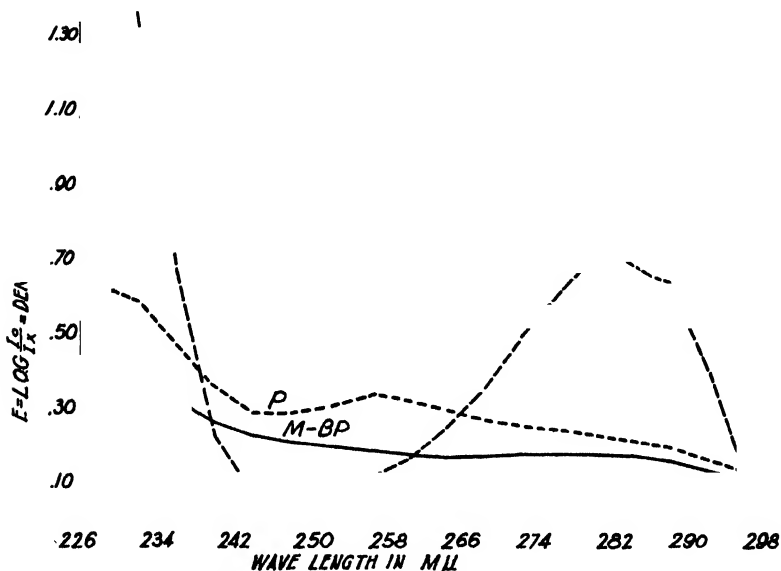


FIG. 4. Comparison of the ultraviolet absorption curve of a standard estriol solution (Curve S) with the curves representing the amounts recovered from identical aliquots by the Pincus modification of the Mather partition (Curve P) and by the Mather-Bachman and Pettit partition (Curve M-BP).

benzene quantitatively. These findings also raise a question with regard to the efficacy of sodium carbonate for the quantitative separation of estriol from estrone and estradiol.

It is clear from the studies which have been described that one must submit to reevaluation the methods currently used for the partition and assay of estrone, estradiol, and estriol. The fact that ultraviolet spectrophotometry provides a sensitive method for the quantitative determination of chemically pure estrogens makes it a feasible means for checking the accuracy of the partition methods, if one eliminates or reduces to an acceptable minimum the optical density of the background material.

### *Studies on Ultraviolet Absorption of Substances Comprising Background Material*

*Experimental Procedure*—In view of the foregoing findings, and on the basis of preliminary exploratory experiments, it seems likely that the background material, which interferes with a quantitative interpretation of the estrogen absorption curves, consists entirely of substances used in the extraction and partition procedure; *i.e.*, reagents and their impurities. Consequently, a study was made of the absorption characteristics of these reagents and their values for  $E$  at 280  $m\mu$ . The experimental conditions under which the reagents were used originally were duplicated carefully. Included in these observations were  $\text{Na}_2\text{HPO}_4$  and  $\text{CCl}_4$  (*cf.* below) for reasons which will appear later on in this communication.

It is well known that petrolatum, rubber, and lubricating greases exhibit absorption in the ultraviolet range. It was suspected early in these experiments that stop-cock grease was carried along into the extracts by the organic and aqueous alkaline solvents used in the partition and extraction of estrogens. An effort was made, therefore, to determine whether or not these lubricants interfere with the quantitative determination of the estrogens. Aqueous alkaline solutions were passed through stop-cocks lubricated with Lubriseal, Trutest, and bentonite-glycerol mixture, respectively. The alkaline solutions were then acidified and extracted with ether. The residues after ether distillation were dissolved in 95 per cent alcohol and subjected to ultraviolet spectrophotometry. Benzene and ether were put through similarly lubricated stop-cocks, and evaporated to dryness subsequently. The residues were taken up in alcohol and then studied spectrophotometrically. The aqueous and organic solvents were used also in testing other substances which belong to classes of compounds that do not show significant absorption in the ultraviolet, but which do exhibit lubricating properties; *e.g.*, powdered agar, granular gelatin, and flaked graphite.

*Results*—Of the reagents commonly used in the extraction and partition of estrogens the following were found to be relatively free of interfering

optical density in the range covered by the ultraviolet absorption curve of the estrogens: alcoholic solutions of the residues of U. S. P. ether, freshly distilled from ferrous sulfate, Merck's redistilled thiophene-free benzene, carbon tetrachloride freed from carbon disulfide, U. S. P. ethyl alcohol, disodium acid phosphate, U. S. P. powdered agar, granular gelatin, flaked graphite, C.P. sulfuric acid.

The successful application of ultraviolet spectrophotometry to the assay of urinary estrogens necessitated substitutions for other reagents and substances exhibiting an optical density of more than 0.1 at 280  $m\mu$ . Agar was adopted instead of the commercial stop-cock lubricants ordinarily used, and  $H_2SO_4$  was substituted for  $HCl$ . The use of filter paper was omitted entirely in view of its contribution to the optical density. Because the absorption curve of the carbonate radical was found to interfere with the measurement of estrogens at 280  $m\mu$ , carbonates were omitted from the procedure for the extraction and partition of estrogens whenever possible. It was for this reason that calcium hydroxide was not used in the removal of peroxides from ether, and that No. 10 mesh glass chips washed with sulfuric acid, water, and ether were substituted for porcelain boiling chips in the distillation of ether from extracts. Moreover, traces of moisture were removed from the residues of ether extracts exclusively by suction in order to avoid the use of anhydrous sodium sulfate, which may contain detectable amounts of carbonate.

*Substitution of Dibasic Sodium Acid Phosphate for Sodium Carbonate in Partition of Estriol from Estrone-Estradiol Fraction*—The foregoing data, considered in conjunction with other findings which suggested that sodium carbonate may be inefficient in the separation of estriol from estrone and estradiol, posed the possibility of substituting sodium acid phosphate for sodium carbonate.

Samples of crystalline estrone, estradiol, and estriol were dissolved in 95 per cent ethyl alcohol to a concentration of 100  $\gamma$  of estradiol per ml., 98  $\gamma$  of estriol per ml., and 103.6  $\gamma$  of estrone per ml. Since the immediate purpose of this experiment was to determine the benzene-sodium carbonate distribution of each estrogen, 5 ml. aliquots of each solution were dried and carried independently through the entire partition procedure. The residues were dissolved in 1.0 ml. of 95 per cent ethyl alcohol, and 100 ml. of thiophene-free benzene were added to each. The benzene solutions were extracted three times with 50 ml. volumes of 0.3  $M$   $Na_2CO_3$ . The benzene phase, containing the estrone-estradiol fraction, was taken to dryness with suction on a warm water bath, and the residue thereof was dissolved in 10 ml. of 95 per cent ethyl alcohol. The aqueous alkaline phase, containing the estriol, was acidified with 6  $N$   $H_2SO_4$  to Congo red and extracted with four 50 ml. volumes of ethyl ether, which was distilled off subsequently.

The residue of the ether extract was dissolved in 5 ml. of 95 per cent ethyl alcohol. Spectrophotometric curves were prepared of the alcoholic solutions of these organic and inorganic phases.

The foregoing experiment was repeated with 0.075 and 0.2 M  $\text{Na}_2\text{HPO}_4$  in place of 0.3 M  $\text{Na}_2\text{CO}_3$ .

TABLE VI  
*Distribution of Estrone, Estradiol, and Estriol between Benzene and Alkali*

Experiment No.	Estrone recovery		Estradiol recovery		Estriol recovery	
	Benzene phase	Alkaline phase	Benzene phase	Alkaline phase	Benzene phase	Alkaline phase
0.3 M $\text{Na}_2\text{CO}_3$						
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	90.8		66.7	31.7	2.1	
2	92.1		68.6	33.9	0	105.6*
3	91.4	2.4	68.5	33.6	0.6	98.9
4	94.9	5.1	70.2	35.7	0	101.7
5	94.7	3.4	64.5	33.3	0	96.0
6	91.5	3.0				
0.075 M and 0.2 M $\text{Na}_2\text{HPO}_4$ †						
1	102.4	0	100.0	0	8.2	90.3
2	98.7	0	102.0	0	4.0	94.8
3	100.4	0	102.0	0	8.6	90.8
4					6.1	91.9
5	101.2	0.6	102.1	0.8	2.7	100.4

\* The range of these values is not an indication of the accuracy of this method of assay. At this point in the partition the reagent control shows a relatively low density reading at 280  $\mu$ , which interferes with the exact quantitative estimation of the estrogen content of these samples. This is of no consequence, inasmuch as the estrogen content of the sample is not tested for until the end of the procedure, when the density of the reagent control is at an optimum level for exact quantitative work. The data in this table are of value, nevertheless, since they disclose large enough deviations to merit attention.

† The difference in pH between 0.075 M and 0.2 M  $\text{Na}_2\text{HPO}_4$  is negligible. The values lie between pH 8.7 and 8.8.

**Results**—The results of the experiments with 0.3 M  $\text{Na}_2\text{CO}_3$  are recorded in Table VI. These data indicate that 0.3 M  $\text{Na}_2\text{CO}_3$  is not a satisfactory substance for the partition of estrogens, previous statements in the literature to the contrary. Approximately one-third of the estradiol is carried over into the alkaline phase with the estriol. This results in a large error in the determination of the estradiol as well as of the estriol.

Reference to Table VI discloses, on the other hand, that 0.075 and 0.2 M

$\text{Na}_2\text{HPO}_4$  achieve an acceptable quantitative separation of estriol from the estrone-estradiol fraction. Since estradiol is not carried into the aqueous alkaline phase, its quantitative recovery is not interfered with. Furthermore, estradiol no longer masks the extent to which estriol is soluble in the alkaline solution. The small fraction of estriol, which remains in the benzene phase, does not affect the final recovery values for either estrone or estradiol, because it is removed later by the discarded  $\text{Na}_2\text{CO}_3$  washings during the Girard separation.

It appears, therefore, that the method in common use for the separation of estriol from the estrone-estradiol fraction is grossly inaccurate. This situation may have been overlooked, because the bioassay method used currently for the measurement of these substances is not quantitative as compared with the micro precision of ultraviolet spectrophotometry.

In view of the foregoing, it seemed essential to check up on the accuracy of the remainder of the method employed in the separation of the estrogens from other urinary materials and from one another.

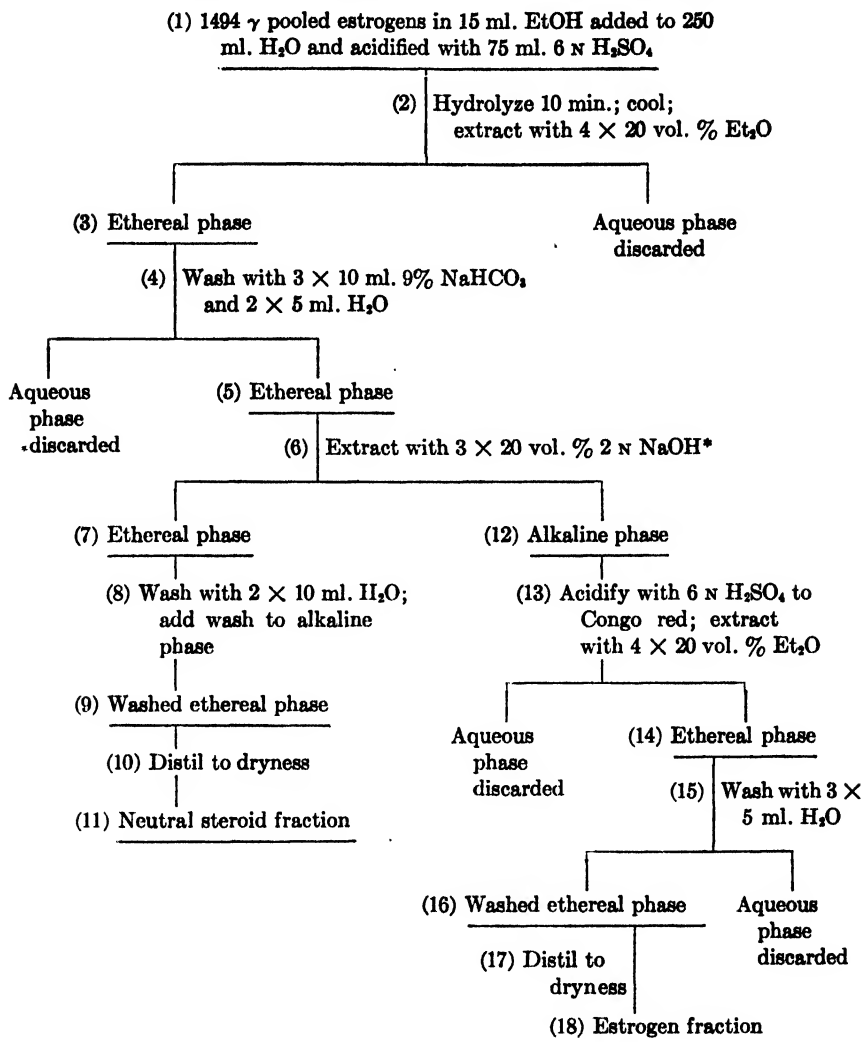
#### *Separation of Phenolic Estrogens from So Called Neutral Steroid Fraction*

In general, estrogens have been separated from androgens by one of two types of method; *viz.*, simultaneous extraction of the three urinary estrogens from an ether or toluene extract of hydrolyzed urine by a given concentration of KOH or NaOH (1, 16-22), or differential extraction of the estrogens by two concentrations of alkali (10, 11, 16, 23). The latter method, or its modification, involves the extraction of estriol by 0.1 N NaOH from ether, and the removal from toluene of the estrone-estradiol fraction by N NaOH. The latter leaves the neutral steroid fraction in the toluene.

Since toluene is known to exhibit interfering absorption over that part of the ultraviolet spectrum which is occupied by the estrogen curves (13), it was inadvisable to use the ether-toluene method for the separation of estrogens from androgens. Consequently, we studied the application of ultraviolet spectrophotometry to the other type of method, which involved the removal of estrogens by NaOH from an ether extract of hydrolyzed urine.

*Experimental Procedure*—In order to duplicate as closely as possible the steps (Table VII) that are involved in the extraction of estrogens from urine samples, an aqueous alcoholic solution of estrone, estradiol, and estriol was prepared and subjected to hydrolysis for 10 minutes with 30 volumes per cent of 6 N  $\text{H}_2\text{SO}_4$  (equivalent to 15 volumes per cent of concentrated HCl). The hydrolysate was cooled rapidly and extracted four times with 20 volumes per cent of ethyl ether. The ethereal extract was washed with three 10 ml. volumes of 9 per cent  $\text{NaHCO}_3$  solution to remove acids, and the washings were discarded. The ether phase was then extracted three

TABLE VII

*Procedure for Separation of Estrogens from So Called Neutral Steroid Fraction*

\* Nineteen experiments were done in all. Three extractions with alkali were used in the first two experiments, six extractions with alkali were carried out in the next twelve experiments, and four extractions were done in the last five experiments. Concentrations of NaOH or KOH varied from 1.5 N to 6 N (see Table VIII).



times with 20 volumes per cent of 2 or 2.5 N NaOH solution. The ether phase was washed twice with 10 ml. volumes of distilled water, and the washings were added to the alkaline phase. The ethereal solution was distilled to dryness, taken up in 4 ml. of 95 per cent ethyl alcohol, and the ultraviolet absorption curve of this solution was determined. The alkaline phase was acidified with 6 N  $\text{H}_2\text{SO}_4$  to Congo red and extracted four times with 20 volumes per cent of ethyl ether. This ethereal extract was washed three times with 5 ml. volumes of distilled water, and the ether removed by distillation. The residue thereof was dissolved in 4 ml. of 95 per cent ethyl alcohol and the ultraviolet absorption curve of this solution was prepared.

*Results*—The results recorded in Table VIII indicate that a significant proportion of the estrogenic hormones, amounting approximately to 26 per cent of the total estrogen content of the initial sample, is left behind in the so called neutral steroid fraction by 2 N or 2.5 N NaOH.

In order to determine which of the three estrogens, or what combination of the three, remained in the organic phase, each of them was subjected to extraction from ether by a variety of concentrations of NaOH ranging from 1.5 to 6 N.

The results of these studies showed that estriol is extracted from ether quantitatively by 1.5 to 2.5 N NaOH. It was apparent, therefore, that the 26 per cent of estrogen lost in the androgen fraction must have consisted either of estrone or estradiol, or both. Because estrone is relatively less soluble in alkali than estriol, other experiments were done in which estrone was extracted from an ethereal solution by 3 and 6 N NaOH. Estradiol, which has an intermediate solubility in alkali, was extracted from an ethereal solution by 3 N NaOH.

The results of this investigation showed that 46 per cent of the estrone and 46 per cent of the estradiol were left in the androgen fraction by the 3 N NaOH, and 81 per cent of the estrone was unextracted by the 6 N NaOH.

One must conclude, therefore, that estrone and estradiol cannot be removed quantitatively from an ethereal extract by four to six extractions with NaOH solutions ranging in concentration from 2 to 6 N. Although it is possible to effect a quantitative separation under these experimental conditions by a considerably greater number of extractions, the latter would render the procedure impractical. Confronted with this impasse, we sought other means to achieve a quantitative removal of estrone and estradiol from an ethereal extract.

*Further Experimental Procedures*—The quantitative removal of estrone and estradiol from an ethereal extract was accomplished through a reduction in the solubility of these estrogens in the organic phase by the addition to the ether of carbon tetrachloride, in which estrogens are considerably less

soluble (24). Thus the distribution of estrogens between the organic and inorganic phases was altered in favor of the latter.

By trial and error it was determined that a 1:18 ratio of ether to carbon tetrachloride results in a quantitative removal of estriol, estrone, and estradiol by *N* KOH. KOH was substituted for NaOH because KOH exhibits a slightly greater alkalinity for the same normality. This is de-

TABLE VIII  
*Separation of Estrogen Fraction from So Called Neutral Steroid Fraction*

Experiment No.	Estrogen	Organic solvent	Alkali	No. of extractions	Alkali	Estrogen in total neutral steroid fraction		Estrogen in estrogen fraction
						vol. per cent	per cent	per cent
1	Pool*	Et <sub>2</sub> O	NaOH, 2 <i>N</i>	3	20	37		
2	"	"	" 2 "	3	20	26		67†
3	"	"	" 2.5 "	6	15	27		76†
4	Estriol	"	" 1.5 "	6	15	0		94‡
5	"	"	" 2.5 "	6	15	0		99‡
6	Estrone	"	" 3 "	6	15	46		51‡
7	"	"	" 6 "	6	15	81		16‡
8	Estradiol	"	" 3 "	6	15	46		55‡
9	Estrone	Et <sub>2</sub> O-CCl <sub>4</sub> , 1:1	" 2.5 "	6	15	41		65‡
10	"	" 1:1	" 2 "	6	15	28		
11	"	" 1:1	KOH, 2 "	6	15	22		
12	"	" 1:1	" 1 "	6	15	17		
13	"	" 1:1	" 0.5 "	6	15	28		
14	"	" 1:9	" 1 "	6	15	0		
15	"	" 1:9	" 1 "	4	15	3.8		96‡
16	Estradiol	" 1:9	" 1 "	4	15	0		96‡
17	Estriol	" 1:9	" 1 "	4	15	0		91‡
18	Pool*	" 1:18	" 1 "	4	50	0.8		
19-25	"	" 1:18	" 1 "	4	50	0		

\* Estrone, estradiol, and estriol in approximately equal amounts.

† Aliquot of ether extract of acidified alkaline phase.

‡ Total estrogen fraction.

sirable, inasmuch as the salt content of the final residues should be kept as low as possible in spectrophotometric work.

It remained to find out whether or not the androgens are carried over into the estrogen fraction, and vice versa, under the foregoing experimental conditions. Accordingly, three groups of experiments were set up in which single specimens of androgens, pools of androgens, and pools of androgens plus estrogens were submitted to the separation procedure.

In Experiment 1, an alcoholic solution containing 4.98 mg. of androsterone was dried by suction in a separatory funnel. The residue thereof was dissolved in ether and carbon tetrachloride, and extracted four times with N KOH. The latter pooled aqueous extract was washed two times with carbon tetrachloride. This wash was treated as the original organic phase and kept separate from it. (Subsequent trials have shown that such a wash does not carry estrogens along with it.) The aqueous fraction, ordinarily containing the estrogens, was then acidified and extracted with ether. The ethereal extract was washed with sodium bicarbonate solution and with water and distilled to dryness by steam. The final residue was dissolved in alcohol, and the solution examined spectrophotometrically. The organic fraction containing the androgens was washed with water (the wash being added to the original aqueous phase) and dried by suction. The residue was dissolved in alcohol in preparation for spectrophotometry. A 5.15 mg. sample of isoandrosterone was treated in an identical manner.

In Experiment 2, a pooled sample of 10.92 mg. of androsterone and 2.2 mg. of dehydroisoandrosterone was subjected to the procedure outlined for Experiment 1. An identical sample was treated likewise, except that it was hydrolyzed for 30 minutes instead of 10 minutes.

In Experiment 3, two identical aliquots of a pooled sample of estrogens and androgens were subjected to the procedure employed in Experiment 1, except for the wash of the aqueous phase which was omitted. Each ml. of this pool contained 10.04 mg. of androsterone, 1.32 mg. of dehydroisoandrosterone, 29.4  $\gamma$  of estrone, 30.0  $\gamma$  of estradiol, and 29.7  $\gamma$  of estriol.

*Results*—The results in Table IX indicate that the foregoing procedure separates androgens from estrogens quantitatively.

#### *Separation of Urinary Estrogens from Other Urinary Phenolic Substances by Steam Distillation*

The foregoing studies with crystalline estrogens have had as their eventual goal the application of the spectrophotometric method to the assay of the estrogens in urine. In this connection, one should recall that the ultraviolet absorption curves characteristic of the estrogens are simulated by phenolic substances, including the urinary phenols, in that they show pronounced absorption over the same range of the spectrum with maximal density in or about the region of 280  $m\mu$  (3, 4, 20). It follows, therefore, that the estrogens must be separated quantitatively from the other urinary phenolic substances before they can be determined quantitatively by the ultraviolet spectrophotometric method.

■ In accordance with equations formulated by Naumann (25) and by Virtanen and Pulkki (25), it can be predicted that steam distillation can be used to effect this separation.

Because of the relative insolubility in water and the low vapor pressure of the estrogenic phenols, they are practically non-steam-volatile. This may be contrasted with the ready solubility and relatively higher vapor pressure of the other urinary non-estrogenic phenols, such as phenol and *p*-cresol, which consequently are steam-volatile. In accord with these theoretical considerations, Dobriner, Lavin, and Rhoads (20) found that estriol cannot be detected spectroscopically in the *steam-volatile* fraction after steam distil-

TABLE IX  
*Separation of Crystalline Androgens from Crystalline Estrogens*

Experiment No.	Hydrolysis	Steroids recovered	
		Estrogen fraction	Androgen fraction
		<i>per cent</i>	<i>per cent</i>
1. Androsterone, 4.98 mg. Isoandrosterone, 5.15 mg.	None	0	
2. Each ml. of pooled sample contained androsterone 0.905 mg., dehydroisoandrosterone 0.100 mg.	"	0	112*
Aliquot 1	10 min.	0	106*
" 2	30 "	0	113*
3. Each ml. of pooled sample contained androsterone 10.04 mg., dehydroisoandrosterone 1.32 mg., estrone 29.4 $\gamma$ , estradiol 30.0 $\gamma$ , estriol 29.7 $\gamma$			
Aliquot 1	None	107*	109*
" 2	"	104*	112*

\* These spectrophotometric curves disclose small traces of a residue of carbon tetrachloride, which is difficult to get rid of at this point, but which is apparently removed later on in the procedure. This may account for the experimental error represented by the difference between 100 per cent and these values. The latter cannot be attributed to the inefficient separation of estrogens and androgens, inasmuch as there is no indication of the presence of a steroid in the contour of the curve representing the difference between the reagent control and the recovered androgens or estrogens.

lation, whereas the absorption bands of phenols, such as phenol and the cresols, are absent from the *non-steam-volatile* fraction after steam distillation. They did not attempt a quantitative recovery of the estriol, which may be a necessary step, inasmuch as the steam distillation took place in an acid medium in which destruction of estrogens might have occurred.

The present report deals with two aspects of this problem: (1) to determine the effect of steam distillation on the quantitative recovery of crystalline estrone, estradiol, and estriol; (2) to ascertain whether or not

the steam-volatile phenols of hydrolyzed male urine can be separated completely from the non-steam-volatile fraction by steam distillation.

*Experiment 1*—Approximately equal amounts of chemically pure crystalline estrone, estradiol, and estriol were dissolved in 95 per cent ethyl alcohol to make a solution containing 99.6  $\gamma$  of pooled estrogens per ml. 15 ml. of this solution were placed in a small boiling flask and dried by suction. The residue was dissolved in 0.5 ml. of 95 per cent ethyl alcohol, diluted with 5 ml. of distilled water, and steam-distilled in an all-glass still to 500 ml. of distillate. The steam distillation having been completed, the aqueous solution remaining in the flask was dried by suction, and the residues dissolved in 15 ml. of 95 per cent ethyl alcohol. An absorption curve was determined on this solution.

*Results*—Steam distillation permitted the quantitative recovery of the three estrogens with which the experiment was started. Comparison of the absorption characteristics of the pooled sample, which had been subjected to steam distillation, with that of an identical untreated aliquot disclosed a difference of only 0.6 per cent when measured at 280  $m\mu$ .

*Experiment 2*—1 liter of male urine was hydrolyzed with 15 volumes per cent of concentrated hydrochloric acid for 10 minutes and extracted with 20 volumes per cent of ethyl ether (24). The ether extract was washed with 9 per cent sodium bicarbonate solution, and the androgenic fraction separated from the estrogenic fraction by  $N$  KOH. The alkaline estrogenic fraction was acidified and extracted with ethyl ether. The ether was distilled off, and the residue dissolved in 50 ml. of 95 per cent ethyl alcohol. The equal aliquots of this solution were diluted 10-fold in order to reduce the optical density of the latter to a readable level. This dilution factor served also to eliminate from spectrophotometric consideration an already relatively low content of estrogen, so that this extract contained essentially only non-estrogenic urinary phenols. One aliquot was steam-distilled by the method described for Experiment 1. Both aliquots were tested spectrophotometrically.

*Results*—In accordance with the observations of other investigators (3, 4, 20), we have found that urinary phenols, other than the estrogens, are characterized by ultraviolet absorption curves, which resemble closely those of estrone, estriol, and estradiol. Curve A, Fig. 5, represents the ultraviolet absorption of the total phenolic fraction of a 1000 ml. sample of male urine. The shape of this curve, and the fact that it exhibits minimal density at 250  $m\mu$  and maximal density at 280  $m\mu$ , endow it with the general and specific absorption characteristics of the urinary phenols, which include the estrogens. Curve B represents the absorption of this urinary extract after steam distillation. It is clear from the shape of Curve B that the substances which produced the typical phenolic absorption are steam-vola-

tile. This was verified by calculating the difference between Curves A and B, the results of which are plotted in Curve C. Curve C is typically phenolic. Although Curve B, which represents the non-steam-volatile fraction, does not disclose the presence of phenolic substances, this does not constitute proof of their absence, since the ratio of the density of the urinary background material to that of the estrogen content determines whether the typical estrogen absorption curve is detectable. It should be noted that the foregoing experiment was not meant to be quantitative. It

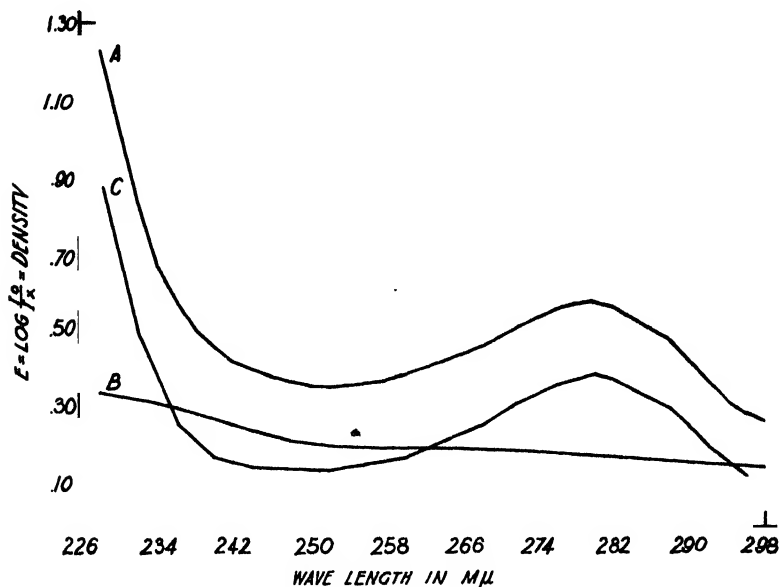


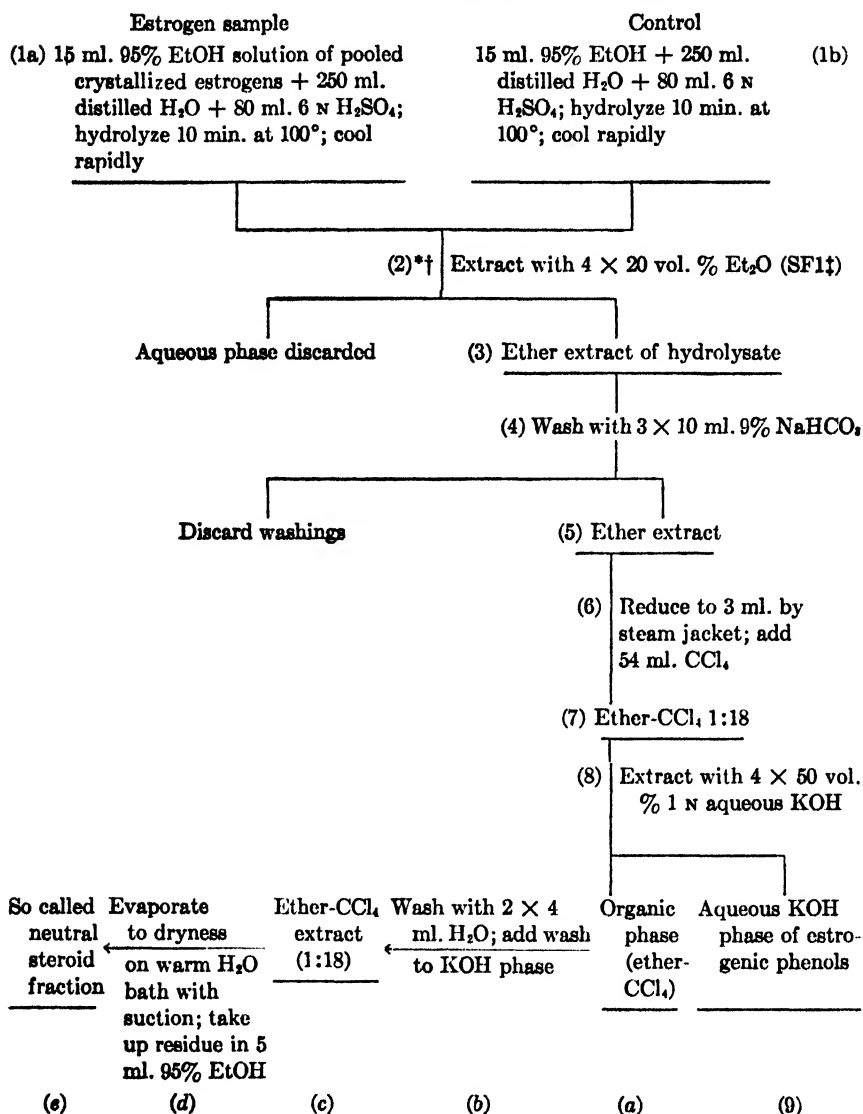
FIG. 5. Steam distillation of extract of male urine. Curve A, total phenolic fraction; Curve B, non-steam-volatile fraction; Curve C, steam-volatile fraction (Curve A minus Curve B).

was devised only for the purpose of demonstrating what type of absorption curve is characteristic of the steam-volatile urinary phenols.

#### *Micro-Girard Separation of Estrone from Estradiol*

Having developed a satisfactory quantitative method for the separation of estrogens from androgens, and estriol from the estrone-estradiol fraction, it remained for us to check the accuracy of the micro-Girard method, which has to do with the separation of estrone from estradiol. Sulfuric acid was not substituted for hydrochloric acid, as recommended in other parts of the extraction and partition procedure, because its use in the Girard reaction is disadvantageous. During this reaction sulfuric acid causes the formation

TABLE X

*Extraction and Separation of Estrogens from Androgens*

\* Quantitative check by ultraviolet curve on aliquot of ether extract.

† Quantitative check by ultraviolet curve of fifth ether extract (for completeness of extraction).

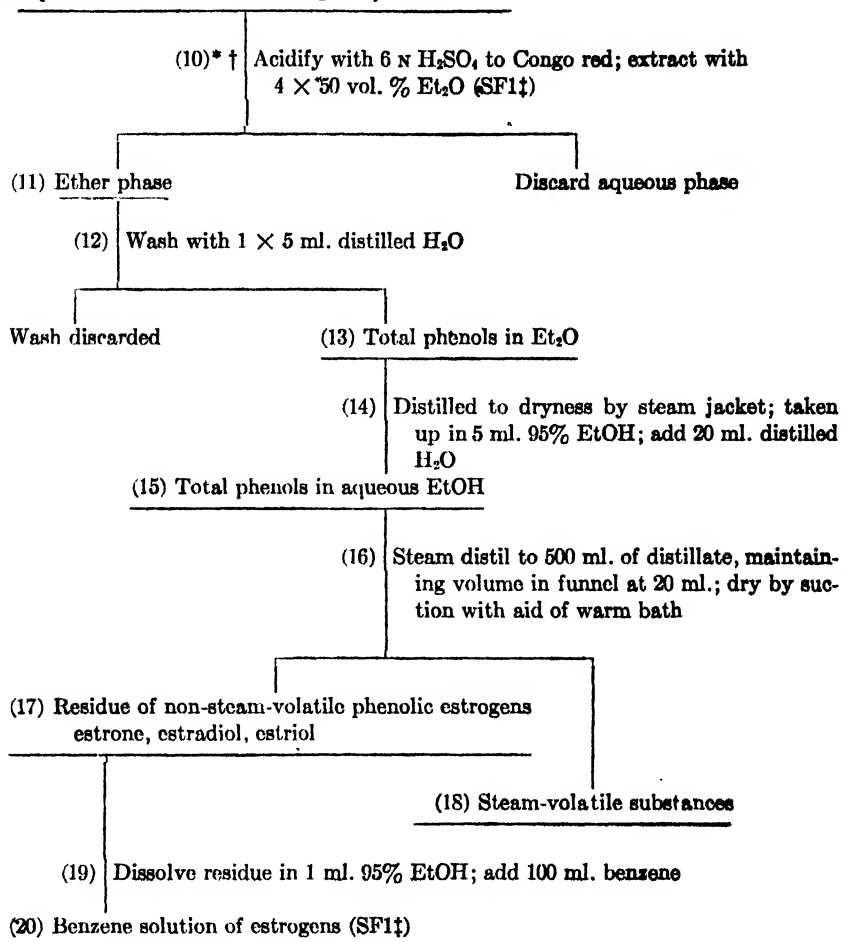
‡ SF1 represents extraction in a 500 ml. separatory funnel.

of more interfering background material than is encountered with the use of hydrochloric acid.

TABLE XI

*Separation of Non-Steam-Volatile Estrogenic Phenols from Steam-Volatile Substances*

(9) Aqueous KOH extract of estrogenic phenols



\* Quantitative check by ultraviolet curve on aliquot of ether extract.

† Quantitative check by ultraviolet curve of fifth ether extract (for completeness of extraction).

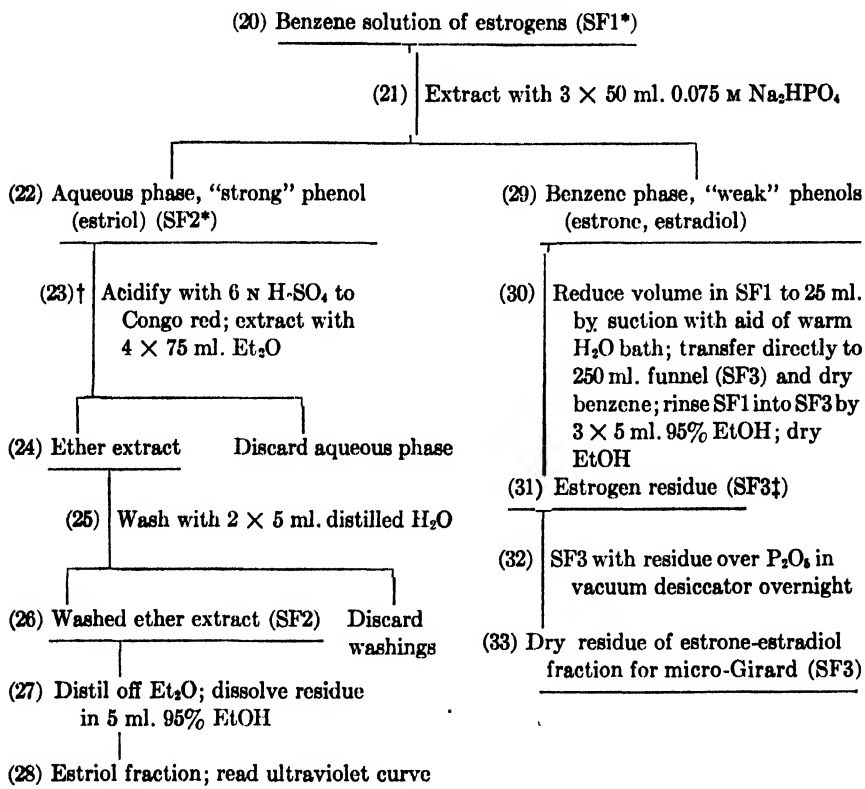
‡ SF1 represents extraction in a 500 ml. separatory funnel.

*Experimental Procedure*—Exploratory experiments to determine the accuracy of the Pincus-Pearlman modification (26) of the micro-Girard



method were carried out on three samples of estrogens. A pooled sample of 500  $\gamma$  each of estrone, estradiol, and estriol and a single sample of 500  $\gamma$  of estradiol were subjected to separation at the point of the benzene-phosphate partition; the benzene fraction was carried through the micro-Girard pro-

TABLE XII  
*Separation of Estriol from Estrone-Estradiol Fraction*



\* SF1 and SF2 represent two 500 ml. separatory funnels.

† Quantitative check by ultraviolet curve of fifth ether extract (for completeness of extraction).

‡ SF3 represents extraction in a 250 ml. separatory funnel.

cedure subsequently. A third pooled sample containing 500  $\gamma$  each of estrone and estradiol was subjected to the micro-Girard procedure only. The concentration of estrogen in each instance was about 100  $\gamma$  per ml.

**Results**—The results of these experiments were as follows:

92 per cent of the estriol from the pooled sample of three estrogens was recovered from the phosphate phase. Of the estrogens in the benzene

TABLE XIII

*Modified Micro-Girard Separation of Estrone from Estradiol*

(33) Dry residue of estrone-estradiol fraction (SF3*)	
(34) Add 2 ml. glacial acetic acid, then 400 mg. Girard's Reagent T. Stopper funnel with adapter fitted with CaCl <sub>2</sub> tube; heat on H <sub>2</sub> O or glycerol bath at 90-100° for 20 min.; cool in desiccator; add 60 ml. ice-cold distilled H <sub>2</sub> O, then 14 ml. chilled 10% NaOH; mix; extract with 4 × 50 ml. Et <sub>2</sub> O†	
(35) Ether phase, estradiol fraction (SF3)	(42) Aqueous alkaline phase, estrone fraction
(36) Wash with 1 × 20 ml. ice-cold distilled H <sub>2</sub> O; add wash to aqueous phase	(43) Acidify with 12 ml. concentrated HCl; keep at room temperature for 2 hrs.; extract with 3 × 50 ml. Et <sub>2</sub> O in SF4††
(37) Washed ether extract	
(38) Wash with 1 × 20 ml. 2.5% Na <sub>2</sub> CO <sub>3</sub> and 3 × 10 ml. H <sub>2</sub> O	<div style="display: flex; justify-content: space-between;"> <span>Aqueous phase discarded</span> <span>(44) Ether extract</span> </div>
(39) Ether extract (SF3)      Aqueous phase discarded	
(40) Distil to dryness; dissolve residue in 5 ml. 95% EtOH	(45) Wash with 1 × 20 ml. 2.5% Na <sub>2</sub> CO <sub>3</sub> and 3 × 10 ml. distilled H <sub>2</sub> O
(41) <u>Alcoholic solution of estradiol</u> (Read ultraviolet curve)	(46) Ether extract (SF4)      Aqueous phase discarded
	(47) Distil to dryness; dissolve residue in 5 ml. 95% EtOH
	(48) <u>Alcoholic solution of estrone</u> (Read ultraviolet curve)

\* SF3 and SF4 represent extraction in a 250 ml. separatory funnel.

† Quantitative check by ultraviolet curve of fifth ether extract (for completeness of extraction).

phase, 100.4 per cent of the estrone and 103 per cent of the estradiol were recovered after the Girard separation.

97 per cent of the estradiol and 102 per cent of the estrone were recovered

from the pool of these two estrogens after they had been subjected to the Girard procedure only.

TABLE XIV  
*Recovery of Crystalline Estrogens from Aqueous Alcoholic Solution by Proposed New Procedure*

Experiment No.		Amount added	Remainder after removal of aliquots	Amount recovered	
		$\gamma$	$\gamma$	$\gamma$	per cent
Estrone	1	504	325	312	96.0
	2	539	464	381	82.0*
	3	539	477	444	93.0
	4	539	477	441	92.0
	5	535		490	91.5
	6	535		498	93.0
	7	535		535	99.9
	8	535		506	94.6
Estradiol	1	500	323	296	91.6
	2	513	441	358	81.0*
	3	513	454	436	96.0
	4	513	466	392	84.0*
	5	495		470	95.0
	6	495		479	96.7
	7	495		495	100.0
	8	495		490	99.0
Estriol†	1	540		491	91.0
	2	540		502	93.0
	3	540		509	98.0
	4	540		506	93.7
	5	540		517	95.8

\* Although not a part of the methodology, it was deemed essential to check at strategic points the accuracy of the extraction and partition procedure by removing and assaying quantitatively aliquot portions of the estrogen pool. The sum total of the estrogens removed during these periodic checks was subtracted from the amount originally added, and the remainder was used as a basis for comparison. This added experimental maneuver probably accounts for the occasional low recovery values for estrone and estradiol.

† Prior to these experiments, six determinations were done in which the recovery of estriol varied from 82.9 to 89 per cent. Since higher recovery values were obtained consistently by increasing the number of extractions in the benzene-phosphate partition from two to three, and lower values were found consistently when the number of extractions was limited to two, it is assumed that the low values were due to incomplete extraction of estriol from the estrone-estradiol fraction.

Of the estradiol that was carried through the benzene-phosphate partition, none was found in the phosphate phase, in which only estriol is

extracted under these conditions. After the Girard treatment, 93 per cent of the estradiol was found in the non-ketonic fraction, in which it is to be expected, and 5 per cent was lost in the ketonic fraction where it would have interfered with the determination of the estrone ordinarily. This separation was carried out with three 50 ml. volumes of ether. Further investigation disclosed that a fourth ether extraction obviated this 5 per cent loss of estradiol. Several attempts to detect estradiol in a fifth ether extraction failed to disclose its presence, even with the sensitive spectrophotometric technique.

In accordance with our general policy of reducing the number of transfers of material from one piece of apparatus to another, all of the steps up to the actual separation of estrone from estradiol were conducted in a single 250 ml. separatory funnel, which was fitted with a straight adapter closed with a  $\text{CaCl}_2$  tube. The funnel was heated in a horizontal position on a water bath at 90–100° for 20 minutes, during which time it was rotated through 180° every 5 minutes. The neck of the funnel was protected against condensation of steam by a cardboard guard. A glycerol bath was found to be more satisfactory than the water bath. Because of the size of the funnel, it was necessary to increase the volume of glacial acetic acid from 0.5 to 2 ml., with proportionate increases in the amounts of the other reagents in the Girard procedure.

*Essentially New Method for Extraction and Partition of Crystalline Estrone, Estradiol, and Estriol, and Their Quantitative Assay by Ultraviolet Spectrophotometry*

A quantitative ultraviolet spectrophotometric method for the assay of estrogens was developed on the basis of the data which have been recounted in detail throughout this communication. The new features of this method included the following: (a) separation of the androgens from the estrogens by equilibration between ether-carbon tetrachloride (1:18) and  $\text{N KOH}$ ; (b) partition of the estrogens into strongly and weakly phenolic fractions by the use of benzene and dibasic sodium acid phosphate; (c) the use of specially designed apparatus which makes it possible to reduce to a minimum the number of transfers of extracts and residues;<sup>1</sup> (d) application of

<sup>1</sup> We are indebted to Dr. W. Dean Fraser for the design of a special adapter which was used to connect an all-glass steam distillation apparatus with a 500 ml. separatory funnel, thus obviating the necessity of transferring the residue to a boiling flask. Furthermore, a steam jacket, designed to be used on a 500 ml. separatory funnel, enabled us to reduce the volume of ether extracts directly from the funnel in which the extraction was made. Particular attention should be directed to the fact that the extraction and partition procedure for each estrogen was performed in a limited amount of glassware, which consisted of one boiling flask in which the initial hydrolysis was done, two 500 ml. separatory funnels, one 250 ml. separatory funnel, and four Erlenmeyer flasks.

the sensitive ultraviolet spectrophotometric method of assay for the quantitative determination of crystalline estrone, estradiol, and estriol; (e) adoption of agar as the lubricant for stop-cocks of the separatory funnels, because it does not act as an interfering substance from a spectrophotometric view-point.<sup>2</sup>

*Integrated Experimental Evidence Bearing on Accuracy of New Method*—Each of eight pooled samples of an aqueous alcoholic solution of crystalline estrone, estradiol, and estriol and three single samples of estriol were subjected to the entire procedure outlined in the flow sheets of Tables X to XIII. The pooled samples contained approximately 500  $\gamma$  of each estrogen. The exact amounts may be ascertained by reference to the detailed data

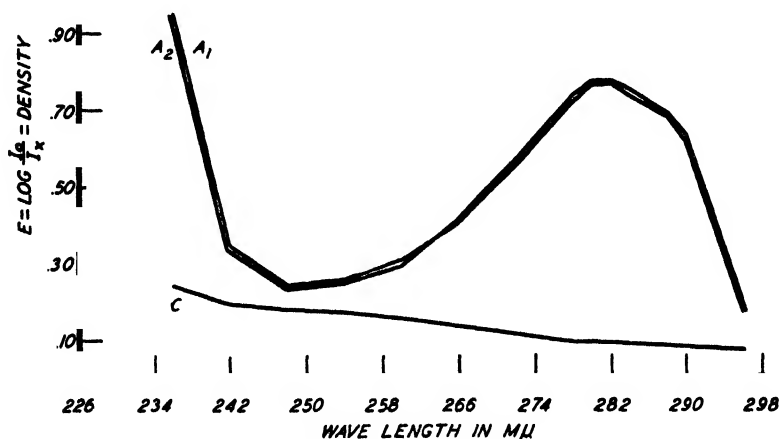


FIG. 6. Recovery of crystalline estradiol from pool of estrone, estradiol, and estriol. Curves A<sub>1</sub> and A<sub>2</sub>, estradiol fractions from duplicate pooled samples; Curve C, reagent control subjected to the identical procedure.

(Table XIV). Reagent controls were set up for each of these experiments in order to correct for the background material. The density value of the curve of the reagent control was subtracted from that of the corresponding estrogen curve at 280  $m\mu$ .

*Results*—Analysis of the ultraviolet absorption curves of the final residues of each of the estrogens indicates that the results of these experiments

<sup>2</sup> The agar was used in the form of a thin, soft jelly made by adding 75 ml. of distilled water to 3 gm. of powdered agar. The mixture is heated in an oven at 110° for 30 minutes and then allowed to stand covered at room temperature for several hours. This substance remains soft and ready for use if it is packed into collapsible tubes. Agar has the additional advantage of being easily removed from stop-cocks with warm water. If it dries on the stop-cock when the funnel is in use, it may be softened by introducing a drop or two of water to the surface by capillary action at each end of the stop-cock.

are reproducible. Reference to Fig. 6 discloses the quantitative accuracy with which estradiol may be recovered from equal aliquots of a pooled sample of estrone, estriol, and estradiol. Identical experiments aimed at the quantitative recovery of estrone and estriol, respectively, yielded equally satisfactory results. The same high degree of accuracy, *i.e.* reproducibility, was achieved in experiments designed to study the optical density of the reagent controls for each of these estrogens. Fig. 7 illustrates this point for estradiol. Although the absorption curves of the reagent controls are of the same order of magnitude, it is advisable to run a reagent control on each determination.

The recovery of estrogens subjected to the foregoing procedure is quantitative, and the results are consistent. Estrone and estradiol were each

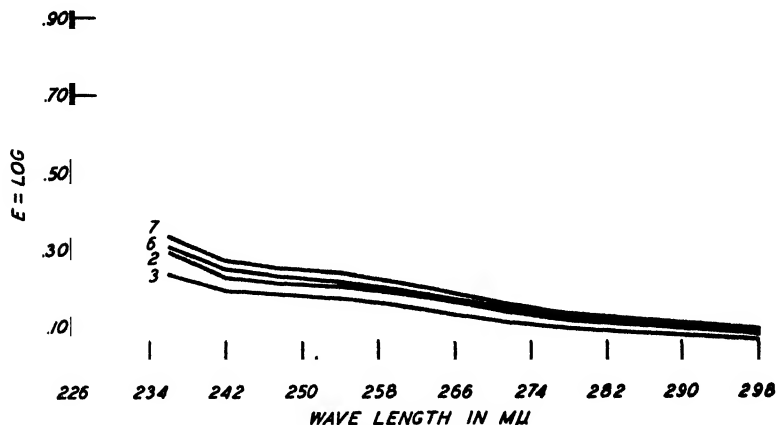


Fig. 7. Reagent controls subjected to the procedure for extraction and partition of estradiol (see Tables X to XIII); four different experiments.

recovered to the extent of 92 to 100 per cent, and estriol to the extent of 91 to 98 per cent.

#### SUMMARY

1. Critical analysis of the literature on data obtained by methods in current use for the partition and assay of urinary estrogens discloses that the quantitative accuracy claimed for them is not in accord with the results on which this contention is based.

2. Restudy of the crucial steps contributing to the inaccuracies of presently accepted methods led to the adoption of the following new features: (a) separation of the androgens from the estrogens by equilibration between ether-carbon tetrachloride (1:18) and *N* KOH; (b) partition of the estrogens into strongly and weakly phenolic fractions by the use of benzene

and dibasic sodium acid phosphate (0.3 M  $\text{Na}_2\text{CO}_3$  is not an efficient solvent for this separation, because one-third of the estradiol is carried over into the estriol fraction); (c) the use of specially designed apparatus which makes it possible to reduce to a minimum the number of transfers of extracts and residues; (d) application of the sensitive ultraviolet spectrophotometric method of assay for the quantitative determination of crystalline estrone, estriol, and estradiol.

3. When subjected to the new procedure, crystalline estrogens were recovered quantitatively and the results were consistent.

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# PLASMA INORGANIC IODIDE AS A HOMEOSTATIC REGULATOR OF THYROID FUNCTION\*

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The enzymatic conversion of iodide to thyroxine and diiodotyrosine is one of the mechanisms enabling the thyroid gland to concentrate iodine. It will be shown in the present study that this mechanism is controlled by the level of plasma inorganic iodine.<sup>1</sup> Organic binding of iodine within the gland can be almost completely blocked by raising the level of plasma inorganic iodine above a certain critical level, which for the rat amounts to about 20 to 35  $\gamma$  per cent. The inhibition, however, is not permanent, for as soon as the level of plasma inorganic iodine falls below this critical range, the gland again resumes its function of organic binding of iodine. These results, therefore, suggest that plasma inorganic iodine acts as a homeostatic regulator in the formation of the thyroid hormone. This regulator probably serves to prevent the formation of excessive amounts of hormone by the gland when the body is suddenly flooded with iodine.

## EXPERIMENTAL

Long-Evans rats weighing 175 to 225 gm. were used throughout. They were injected intraperitoneally with various amounts of iodine in the form of KI labeled with I<sup>131</sup>. In no case was the dose of radioactivity great enough to produce deleterious effects on the gland during the intervals studied.<sup>2</sup> Blood was removed from the heart while the rats were anesthetized with sodium pentobarbital (20 mg. per rat). Equal amounts of plasma from each of five or six animals were pooled, and 2 cc. of plasma were used for duplicate chemical determinations of total plasma iodine (2).

The thyroid glands of the rats used in this investigation weighed  $10.8 \pm 1.9^3$  mg. per 100 gm. of body weight. Each gland contained approximately 5 to 10  $\gamma$  of iodine.

The thyroid gland of each rat, after it had been rapidly excised and weighed, was ground with 1 cc. of cold 10 per cent trichloroacetic acid in

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† Rosenberg Fellow of the University of California.

<sup>1</sup> A preliminary report of some of the data presented here has appeared (1).

<sup>2</sup> Feller, D. D., Taurog, A., and Jones, H. B., unpublished observations.

<sup>3</sup> Standard deviation =  $\sqrt{(\sum_i (x_i - \bar{x})^2)/n}$



an all glass homogenizer. The precipitate obtained by centrifugation was then washed twice with 2.5 cc. of 5 per cent trichloroacetic acid. The supernatants were combined for determination of radioactivity; this fraction contains only the inorganic iodine of the gland. The precipitate was dissolved in a minimal amount of 2 N NaOH on a steam bath, and a suitable aliquot was analyzed for radioactivity; this trichloroacetic acid-insoluble fraction of the gland contains only organically bound iodine.

The absolute amount of the *injected* iodine recovered in a given fraction of the thyroid gland, either total or organically bound, was obtained by multiplying the numerical proportion of the injected radioactivity recovered in that fraction by the micrograms of  $I^{127}$  injected into the rat. Thus, a recovery of 5 per cent of the administered  $I^{131}$  in a fraction prepared from the thyroid of a rat that had been injected with 50  $\gamma$  of  $I^{127}$  would mean that  $0.05 \times 50$  or 2.5  $\gamma$  of the *injected*  $I^{127}$  had accompanied it. By the use of this labeling procedure, the separation of the amount of injected  $I^{127}$  that entered a gland from that present before the injection becomes quite simple. It circumvents the difficulty in making such a separation in glands with variable iodine contents.

In order to test the separation of the gland's iodine into organic and inorganic fractions by the procedure described above, several thyroids were excised from rats and thoroughly ground with 1 cc. of 10 per cent trichloroacetic acid containing 10  $\gamma$  of  $I^{127}$  as KI labeled with  $I^{131}$ . The precipitate was washed as described above. Judging from the amount of radioactivity found in the trichloroacetic acid-insoluble fraction, only 0.05 per cent of the added iodide was recovered in what is termed here the organic fraction.

### Results

Five levels of iodine administration were studied, namely 10, 50, 100, 200, and 500  $\gamma$ , and the results obtained in these five experiments are recorded in Figs. 1 to 5. With the exception of the 10  $\gamma$  dose, the amounts injected greatly exceeded the iodine contents of the thyroids of the rats used here. In each experiment twenty-five to thirty rats were used, all of which were injected with the same amount of labeled  $I^{127}$ . Each thyroid value shown in the figures represents the average of five or six closely agreeing measurements obtained from as many rats.

*Administration of 10  $\gamma$  of  $I^{127}$* —Both total and organic iodine of the thyroids rose rapidly after the injection (Fig. 1). In 2.5 hours, 2  $\gamma$  of the injected iodine had entered the gland, and at this early interval 95 per cent of it was already in the organic form. The maximum amount of iodine (approximately 6  $\gamma$ ) was found at the 5 hour interval; this represents about 60 per cent of the administered iodine.

Not only at the very earliest interval but at all subsequent intervals up to 50 hours after the injection practically the entire amount of the injected iodine acquired by the gland was organically bound.

At the intervals examined, the total plasma iodine in the rats did not

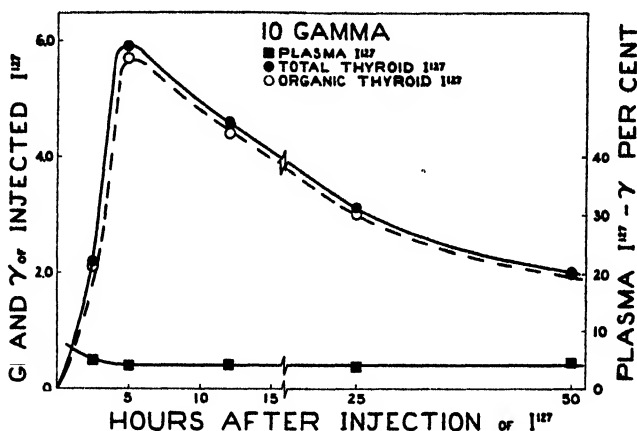


FIG. 1. Changes in plasma and thyroid iodine with time, following the injection of 10  $\gamma$  of iodine in rats.

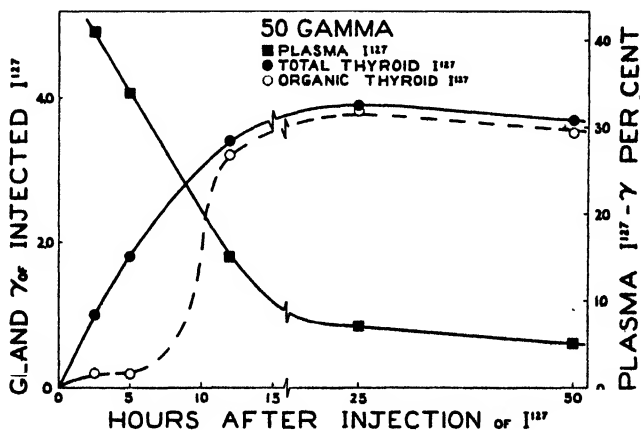


FIG. 2. Changes in plasma and thyroid iodine with time, following the injection of 50  $\gamma$  of iodine in rats.

attain values in excess of 5  $\gamma$  per cent. It should be recalled that approximately 3  $\gamma$  per cent of this total iodine is protein-bound, whereas the remainder is inorganic iodine (2).

*Administration of 50  $\gamma$  of  $I^{127}$* —The results shown in Fig. 2 were unexpected, for less of the injected iodine was found in the thyroids of rats

that received 50  $\gamma$  of  $I^{127}$  than in those of rats injected with 10  $\gamma$ . Even more surprising was the finding that during the first 5 hours (Fig. 2) most of the accumulated iodine was *not organically bound*. Not until after 5 hours did the gland begin to convert significant amounts of the injected iodide into organic forms. Thus at 12, 25, and 50 hours after the injection, 3.4, 3.9, and 3.7  $\gamma$  of the injected  $I^{127}$  were found in the gland, and 95 per cent of these amounts was in the organic form.

During the time when no organic iodine was being formed, the values for plasma iodine exceeded 25  $\gamma$  per cent.

*Administration of 100  $\gamma$  of  $I^{127}$* —Two separate effects produced by the injection of 100  $\gamma$  of iodine are brought out in Fig. 3: (1) inhibition of organic binding of the injected iodine by the thyroid gland during the early

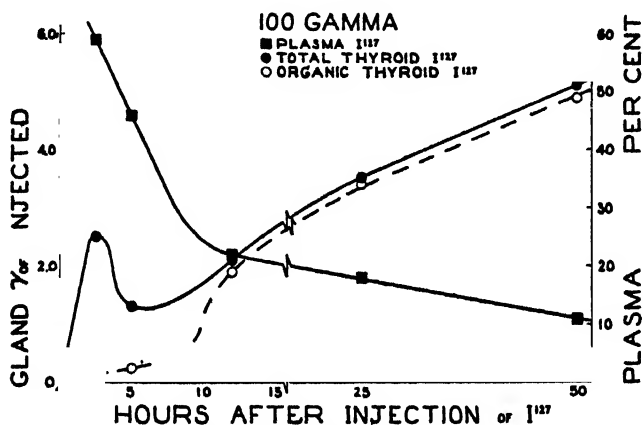


FIG. 3. Changes in plasma and thyroid iodine with time, following the injection of 100  $\gamma$  of iodide in rats.

hours, and (2) early concentration of the injected iodine in the gland in *non-organic form*.

Here, as in the preceding experiment, significant amounts of organic iodine were not synthesized in the gland for the first 8 to 9 hours, during which time the level of plasma iodine exceeded 25  $\gamma$  per cent.

Table I shows quite definitely that the normal gland possesses a mechanism for concentrating iodine that is not dependent upon its organic conversion. During the first 6 hours the concentration of iodine in the gland appeared to parallel roughly that in plasma (Fig. 3). A concentration ratio of approximately 100 to 300 seems to have been maintained during this time between gland and plasma inorganic iodine.

After 12 hours, when the levels of plasma iodine had receded, a gradual accumulation of the injected iodine occurred in the gland, but this time

94 per cent of the injected iodine present in the gland was organically bound.

*Administration of 200  $\gamma$  of  $I^{127}$* —The more striking effects observed in this experiment were to be expected in view of the high plasma iodine levels attained in the rats. The failure of the gland to convert any of the injected iodine to organic forms during the first 12 to 13 hours is clearly brought out in Fig. 4. Although in 2.5 hours 3.4  $\gamma$  of the injected iodine were present in the gland, practically all of it was inorganic iodide. The early accumulation of the injected iodine in the thyroid and its loss reflect the changing

TABLE I

*Iodine-Concentrating Capacity of Thyroid Gland While Organic Binding of Iodine Is Inhibited*

Dose injected	Interval	Plasma iodine	Gland inorganic $I^{127}$ Plasma inorganic $I^{127}$
$\gamma$	hrs.	$\gamma$ per cent	
50	2.5, inhibited*	41	100
	5, inhibited	34	250
	7, escape†	29	200
100	2.5, inhibited	59	210
	5, inhibited	46	130
	9, escape	27	190
200	2.5, inhibited	108	160
	5, inhibited	93	100
	13, escape	20	150
500	2.5, inhibited	330	160
	5, inhibited	253	130
	17, escape	32	220

\* Refers to periods in Figs. 2 to 5 during which organic binding of iodine was inhibited in the gland.

† Interpolated from Figs. 2 to 5; it is the point of inflection in the organic curve and represents the time at which inhibition was released.

level of inorganic iodine in the plasma. During this period the concentration of inorganic iodide in the gland was 100 to 300 times that of plasma (Table I).

When plasma iodine had dropped to concentrations at or below 20  $\gamma$  per cent (approximately 12 to 13 hours after the injection), the mechanism involving the formation of organic iodine in the gland was no longer inhibited and organic iodine rapidly accumulated. At the 50 hour interval about 7  $\gamma$  of iodine had entered the gland and nearly all of it was found organically bound.

*Administration of 500  $\gamma$  of  $I^{127}$* —The results of this experiment, shown

in Fig. 5 and Table I, are one of the clearest demonstrations of the capacity of *normal* thyroid tissue to concentrate iodine in an inorganic form. 10  $\gamma$  of the injected iodine were present in the thyroid gland in 2.5 hours, and

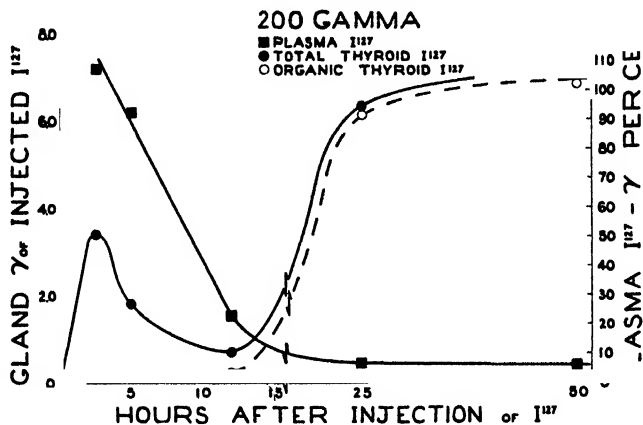


FIG. 4. Changes in plasma and thyroid iodine with time, following the injection of 200  $\gamma$  of iodine in rats.

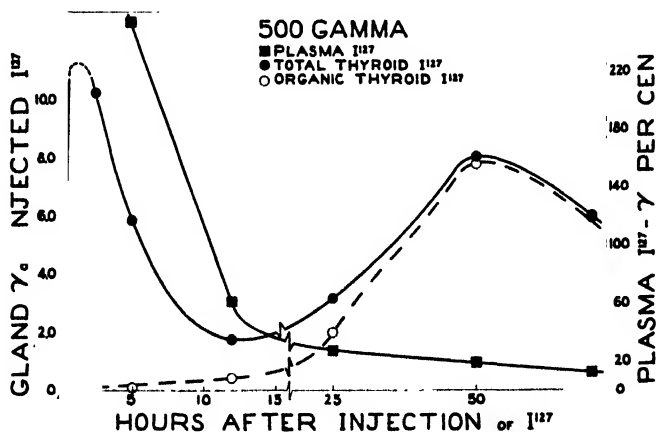


FIG. 5. Changes in plasma and thyroid iodine with time, following the injection of 500  $\gamma$  of iodine. The plasma value for the 2.5 hour interval is not shown; it was 330  $\gamma$  per cent.

practically all of it was recovered in the trichloroacetic acid-soluble fraction prepared from the gland. In 5 hours the amount of the injected iodine in the gland fell to 5.8  $\gamma$ , but again nearly all of it was in the inorganic form. By 12 hours the amount of the injected iodine in the gland fell to 1.7  $\gamma$ ,

nearly all of which was still inorganic. This precipitous rise and fall in the concentration of *inorganic iodine* in the gland during the first 12 hours paralleled the rapid rise and fall in the levels of plasma iodine during this time. Thus at 2.5 hours, when the plasma contained 330  $\gamma$  per cent, the gland contained 10.2  $\gamma$  of the injected iodine; at the 5 hour interval plasma iodine fell to 253  $\gamma$  per cent and the gland iodine to 5.8  $\gamma$ ; at 12 hours plasma and gland contained respectively 61  $\gamma$  per cent and 1.7  $\gamma$ .

The more prolonged inhibition in organic binding of iodine by the gland is in keeping with the very large dose of iodine injected. When 50, 100, and 200  $\gamma$  were administered, nearly all of the injected iodine that had accumulated in the gland at the 25 hour interval was organically bound. This was not the case when 500  $\gamma$  of iodine were injected; even as late as 25 hours only two-thirds of the injected iodine in the thyroid was organically bound. This prolongation of the period of inhibition is related, of course, to the level of plasma iodine. Since we were attempting to determine at precisely what level of plasma iodine the gland is inhibited, it is of interest to note that, when plasma contained 19  $\gamma$  per cent, all of the injected iodine in the gland, namely 8.0  $\gamma$ , was organically bound. Apparently the critical plasma level at which the gland's activity was inhibited was above 19  $\gamma$  per cent.

#### DISCUSSION

The first indication that conversion of inorganic iodide to diiodotyrosine and thyroxine can be blocked in *normal* thyroid tissue by excessive amounts of iodide came from the *in vitro* experiments of Morton *et al.* (3). These workers demonstrated that, when 300 mg. of sheep thyroid tissue were incubated in 3 cc. of a bicarbonate-Ringer's medium containing 1, 5, 10, 15, 20, and 50  $\gamma$  of  $I^{127}$  as inorganic iodide, an inhibitory effect on the conversion of the Ringer's  $I^{127}$  to diiodotyrosine and thyroxine occurred when the amount of  $I^{127}$  exceeded 20  $\gamma$ .

The results of the present investigation confirm and extend the observations of Morton *et al.* (3). They show that the activity of the normal thyroid gland, even *in situ*, is inhibited in the presence of excessive concentrations of inorganic iodide. During the first 6 to 12 hours after the injection into normal rats of 50, 100, 200, and 500  $\gamma$  of iodine, the formation of organically bound iodine is almost completely blocked. The time relations shown in Figs. 2 to 5 suggest that this block in the conversion of the injected iodide to organic forms is related to the level of plasma inorganic iodide. This relation between plasma iodine and thyroid activity is more clearly brought out in Fig. 6, in which the concentrations of plasma iodine were plotted against the amounts of injected iodine that were organically bound in the thyroid glands. Fig. 6 shows that no organic binding of the

injected iodine occurred in our experiments so long as the level of plasma iodine remained above 20 to 35  $\gamma$  per cent, and only when the level of plasma iodine fell below this range did the gland show any capacity for organically binding the injected iodine.

Thus, the inhibitory effect of excessive amounts of iodide upon *normal* thyroid function has now been established *in vivo* as well as *in vitro* and for rat thyroid as well as for sheep thyroid.

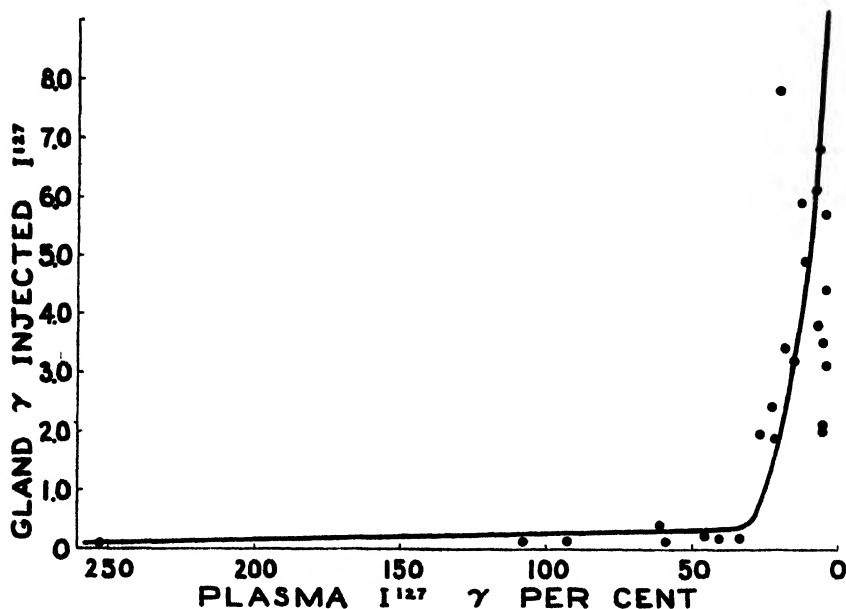


FIG. 6. The relation of the level of plasma iodine to the inhibition of organic binding of iodine in the thyroid gland. The ordinates represent the micrograms of the injected  $I^{127}$  present in the organic fraction of the gland. This figure shows that organically bound iodine can form in the gland only when the concentration of plasma iodine does not exceed 35  $\gamma$  per cent.

Despite an almost complete block in the gland's capacity to bind iodine organically during the first 6 to 12 hours after the injection of massive doses of iodide, the gland does not lose its ability to concentrate iodine at such times. The concentrations of *inorganic iodine* found in the blocked gland<sup>4</sup> were roughly 100 to 300 times those in plasma (Table I). There can no longer be any doubt, therefore, that *even the normal thyroid* possesses a mechanism for concentrating iodine which is not dependent upon its conversion to organic forms. A similar mechanism for concentrating iodine in

<sup>4</sup> The thyroid glands of the rats contained comparatively small amounts of *inorganic* iodine before the injections were made.

the inorganic form has been demonstrated by Vanderlaan and Vanderlaan (4) and by Taurog *et al.* (5) in the thyroids of rats made goitrous by treatment with propylthiouracil. Thus, whether the organic binding of iodine in the gland is blocked by a goitrogenic substance or by excessive amounts of inorganic iodine the thyroid can still concentrate iodine.

It is plausible to assume that the level of plasma iodine is part of a homeostatic mechanism governing hormone synthesis in the gland. This level was found to be approximately 20 to 35  $\gamma$  per cent. Since the concentration of inorganic iodine in the thyroid is approximately 100 to 300 times that in plasma, the inhibitory level inside the gland (undoubtedly the immediate agent responsible for this homeostatic control of hormone synthesis by the gland) amounts to approximately 2 to 10 mg. per cent. Whenever large amounts of iodine are ingested, the animal limits the conversion of inorganic iodine to thyroid hormone and is thereby afforded sufficient time to rid itself of the excess iodine. By means of this homeostatic regulator the formation of toxic amounts of thyroid hormone is prevented.

What is the mechanism by which organic binding of iodine in the *normal* thyroid gland is inhibited by excessive amounts of inorganic iodide? That the inhibition operates by suppressing the secretion of thyrotropic hormone of the anterior hypophysis would appear to be ruled out by the *in vitro* experiments of Morton *et al.* (3). But the possibility that the excess iodide interferes with the action of the thyrotropic hormone already present within the thyroid gland is not excluded. Other explanations, namely (1) iodination of enzymes concerned in the conversion of inorganic iodide to diiodotyrosine and thyroxine and (2) inhibition of the formation of an intermediate in thyroxine synthesis, have been considered by Morton *et al.*

Ever since the introduction of iodine therapy for the treatment of Graves' disease by Plummer in 1923 (6), the mechanism by which iodine brings about a dramatic remission of signs and symptoms in patients suffering from this disease has attracted considerable attention. There can be no doubt that under the influence of therapeutic doses of iodine thyroid hormone in excessive amounts ceases to be delivered to the circulation (7-12). A reversal in the direction in which the hormone is secreted, *i.e.* secretion into the follicle rather than into the blood stream, has been offered as an explanation for this iodine effect upon the hyperthyroid state (13). It has also been postulated that the therapeutic action of iodine is due to the inhibition of the proteolytic enzyme system responsible for the release of the follicular colloid (14). While we shall not attempt to evaluate these theories, we do believe that our findings, even though they deal with normal thyroid tissue, justify the conclusion that an interference in organic binding of iodine by the gland is an integral part of the mechanism by which iodine brings about a remission in Graves' disease. According to this concept,



inhibition of hormone synthesis by excess iodide is a general property of thyroid tissue regardless of its state of activity.

#### SUMMARY

1. Rats were injected with 10, 50, 100, 200, and 500  $\gamma$  of iodine in the form of KI labeled with  $I^{131}$ . The radioactivity served as a measure of the amounts of injected  $I^{127}$  that entered the gland.

2. So long as plasma iodine remained above 20 to 35  $\gamma$  per cent, organic binding of the injected iodide in the gland was blocked. Organic binding of the newly accumulated iodine began to occur only when the level of plasma iodine fell below the critical range of 20 to 35  $\gamma$  per cent.

3. A homeostatic mechanism is postulated in which excessively high levels of plasma iodine inhibit formation of the hormone in the thyroid gland. This mechanism helps to explain the beneficial effect produced in patients suffering from thyrotoxicosis (Graves' disease) when large amounts of iodine are administered.

4. While the organic binding of iodine is blocked, the gland is still able to concentrate iodine. The results presented here thus establish that the normal thyroid gland possesses a mechanism for concentrating iodine that does not depend upon its conversion to thyroxine and diiodotyrosine.

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# STUDIES ON PROTHROMBIN: PURIFICATION, INACTIVATION WITH THROMBIN, AND ACTIVATION WITH THROMBOPLASTIN AND CALCIUM\*

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Prothrombin, thrombin, fibrinogen, thromboplastin, Ac-globulin,<sup>1</sup> fibrinolysin, antifibrinolysin, antithrombin, antithromboplastin, antihemophilia globulin, and heparin are all factors which are known to be a part of the blood clotting mechanism. They function either to provide emergency hemostasis or to prevent intravascular clotting. In the laboratory their activities are recognized and measured, almost exclusively, either by the rate of fibrin formation or by the rate of fibrin disappearance. If such observations and measurements involve only the action of one factor, reliable conclusions can be made with ease. However, when two, three, or more variables are simultaneously involved in a single measurement, it is hazardous to attempt an answer to many of the important questions which require elucidation. Many of the uncertainties and controversies in the literature on this subject are due to the interplay of several variables. Usually when one of the clotting factors is obtained in purified form, experimental variables can be further eliminated; then a period of rapid progress follows and new horizons are seen.

The purification of prothrombin has presented many formidable difficulties, but it has been possible to report steady progress in an uninterrupted effort (1-4) which still requires further work. In the last report (4) material of high purity was obtained inconsistently. The variability in results could not be explained. Furthermore, the products were unstable in solution (5), thus defeating many of the objectives of the work. It is now known that the inconsistent results were due to the assay procedure and not to the method of preparation. The activation of purified prothrombin with thromboplastin and calcium ions can be accelerated by the addition of Ac-globulin (6-8), which, in small amounts, increases not only the rate of activation but also the final thrombin yield (7, 8). The removal of Ac-globulin during the purification processes results in products which are only partially activated in the two stage analytical procedure

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<sup>1</sup> A factor which accelerates the activation of prothrombin.

(9, 10). The prothrombin products obtained by this method (4) are, therefore, more potent than the reports indicate. Usually those preparations with the lowest apparent activity contain the least amount of Ac-globulin. With the aid of a modified two stage technique, in which adequate amounts of Ac-globulin are supplied, the last reported method (4) has been studied extensively. This method of prothrombin preparation appears to be the most reliable of numerous modifications which were attempted in a study involving the preparation of more than 200 prothrombin products. Improvements in the method, reported herein, have been made for the purposes of obtaining stable products and for removing the last traces of Ac-globulin. The modifications do not involve elimination of any further amount of inert material.

The present paper also includes experiments which show that low concentrations of thrombin destroy prothrombin, and medium concentrations of thrombin act on prothrombin in a diphasic manner; first, the prothrombin becomes refractory to thromboplastin and it later regains the ability to form thrombin. High concentrations of thrombin appear to have no effect on prothrombin. Experiments are also outlined which demonstrate that thromboplastin and calcium ions activate prothrombin in the complete absence of Ac-globulin.

### *Purification of Prothrombin*

Prothrombin Product 2 is first prepared exactly as described (4). This entails, briefly, acid precipitation from diluted oxalated bovine plasma, adsorption on magnesium hydroxide, and elution with carbon dioxide under pressure. It was demonstrated that this product could be left overnight at room temperature without immediate loss of prothrombin, but it now appears that, under these conditions, small amounts of thrombin are produced which ultimately affect the stability of the final product. The appearance of this thrombin is avoided by cooling prothrombin Product 2 immediately to 0°. The cooled product may be used at once or may be stored for a period up to 15 hours. Insoluble magnesium salts and fibrinogen are removed by straining through washed gauze. The clarified prothrombin eluate is then fractionated with saturated ammonium sulfate solutions exactly as described (4); this procedure eliminates almost all of the Ac-globulin. The precipitate obtained after saturation to 65 per cent is dissolved in 10 cc. of distilled water and dialyzed against distilled water at pH 7.0 (previous procedure, acidified water) until the specific resistance of the prothrombin solution is from 2000 to 3000 ohms when measured at 5°. This can be accomplished in 1 to 1½ hours by using the dialysis procedures described (4). Following the dialysis period the prothrombin is further purified by acid fractionation at 0°. The pH

is brought to 5.4 by adding 0.1 N hydrochloric acid with constant stirring. A small precipitate which appears is removed by centrifugation. This precipitate contains the greater portion of the remaining Ac-globulin plus a small amount of prothrombin. Prothrombin is then precipitated from the supernatant fluid by adding acid until the pH reaches 4.6. It is removed by centrifugation and dissolved in 10 cc. of distilled water by cautiously adding 0.1 N sodium hydroxide to pH 7.0 to 7.5. The prothrombin may then be obtained in the dried form, without loss of activity, by drying from the frozen state.<sup>\*</sup> The products are almost invariably free of thrombin and as a result are stable in solution for periods of 24 hours or more at room temperature.

#### *Removal of Ac-Globulin Activity from Purified Prothrombin*

The prothrombin products described above contain less than 1 per cent of Ac-globulin by weight.<sup>2</sup> All attempts to remove the remaining Ac-globulin by chemical means have failed. In order to eliminate completely the Ac-globulin activity, a method of heating has been employed. This follows the approach of Owren (11) for the removal of Factor V from prothrombin. It was reported in detail before that Ac-globulin is less stable to heat than prothrombin (8). At 53°, *in neutral distilled water*, prothrombin is fairly stable. Ac-globulin activity disappears precipitously under these conditions (8). In order to assure complete destruction of Ac-globulin activity in the above prothrombin preparations, the latter are subjected to a temperature of 53° for a period of 2 hours. Except for an occasional loss in activity during the 2 hour heat treatment, the prothrombin does not appear to be damaged in any way. After 30 minutes of such treatment it is not possible to detect any Ac-globulin in the products by our method of analysis (8). By this method,  $1.2 \times 10^{-6}$  mg. of purified Ac-globulin can be detected in 1 cc. of test solution.<sup>3</sup> Therefore, the possibility is considered remote that *any* Ac-globulin remains after the full 2 hour heat treatment.

On one occasion some prothrombin was dried from the frozen state prior to the 2 hour heat treatment. The snow-white dry material was then dissolved in saline and heated at 53° for 2 hours. The Ac-globulin

<sup>2</sup> The dry prothrombin products usually weigh about 75 mg. and contain approximately 300 units of Ac-globulin. The best Ac-globulin products average 330 units per mg. of dry weight (8). The latter were estimated to be 50 per cent pure. Therefore, it follows that an average prothrombin product contains 0.45 mg. of Ac-globulin or 0.6 per cent Ac-globulin by weight.

<sup>3</sup> An assay procedure has been developed by which 1/2500 of a unit of Ac-globulin in 1 cc. of test solution can be detected. Since our best Ac-globulin products average 330 units per mg. of dry weight (8), it follows that  $1.2 \times 10^{-6}$  mg. of the preparation can be detected in 1 cc. of test solution.

was destroyed, as anticipated, but an unexpected drop in prothrombin activity was noted. Following that, it was found that prothrombin cannot be heated at that temperature in 0.9 per cent sodium chloride solution. A comparison of typical results obtained on heating prothrombin in distilled water and in 0.9 per cent sodium chloride is presented in Table I. Heating at 53° is possible in aqueous solutions but not in saline solutions.

#### *Inactivation of Prothrombin with Thrombin*

Purified prothrombin, as prepared previously, was not entirely free of thrombin and was not stable at room temperature (5). When dissolved in oxalated bovine plasma, it was stabilized (5). This was thought to be due, in part, to destruction of thrombin impurity by plasma antithrombin. It was, however, impossible to determine to what extent the instability

TABLE I

*Stability of Prothrombin in Distilled Water and in 0.9 Per Cent NaCl at pH 7.2 and at 55°*

Time	Prothrombin content	
	Distilled water	0.9 per cent NaCl
<i>hrs.</i>	<i>units per cc.</i>	<i>units per cc.</i>
0	7200*	7200*
1	7200	5100
3	7000	2300
5	6500*	1400*

\* No detectable thrombin in solution.

could be attributed to thrombin, because prothrombin free of thrombin was not available. In the following experiments it is shown that as little as 1 unit of thrombin associated with 10,000 units of prothrombin is significant in causing prothrombin instability.

In all experiments, the purified prothrombin was dissolved in distilled water. The activity was measured by the modified two stage technique which supplies Ac-globulin (8). The thrombin added to the prothrombin was of high purity, comparable to the material described by Seegers and McGinty (12). This added thrombin activity was always measured and subtracted from the total prothrombin and thrombin activity.

In the first experiment (Fig. 1) the prothrombin, free of thrombin, was dissolved in water. It was stable for 24 hours. At 48 hours a significant decrease in activity had occurred, at which time a trace of thrombin was found to have appeared spontaneously in the solution. After 72 hours 1 unit of thrombin per cc. of solution was present, and the prothrombin concentration had decreased to less than 25 per cent of the original. There-

after it continued to decrease and the thrombin concentration increased until the respective concentrations of prothrombin and thrombin became 400 and 110 units per cc. on the 8th day.

In the second experiment (Fig. 2), 13,000 units of prothrombin were placed in solution with 10 units of thrombin. Within  $2\frac{1}{2}$  hours a significant drop in prothrombin concentration was found. The activity continued to decline and at 19 hours only 1900 units of prothrombin remained. After 72 hours the prothrombin had increased to 2700 units and the thrombin titer had increased to 165 units.

In the third experiment (Fig. 3) 13,000 units of prothrombin were placed in solution with 1000 units of thrombin. In 10 minutes a significant decrease in prothrombin activity was found. Only 3200 units remained

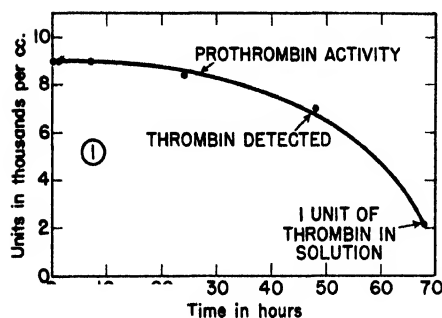


FIG. 1

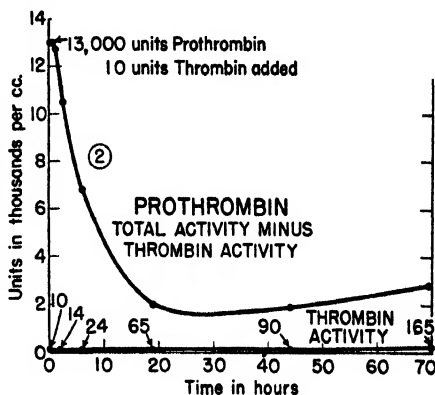


FIG. 2

FIG. 1. Stability of prothrombin, free of thrombin, at 28° in water at pH 7.2.

FIG. 2. Stability of prothrombin at 28° in water solution with 10 units of thrombin per cc. at pH 7.2.

at  $2\frac{1}{2}$  hours, but thereafter the activity began to increase; at 6 hours it was 5500 units, at 19 hours it was 9300 units, and thereafter it decreased slowly to 5450 units in 72 hours. At 19 hours there had occurred a 3-fold increase in the original thrombin added. It increased further to 5800 units, where it remained for the remainder of the experiment. This experiment has been repeated many times. The amount of prothrombin regeneration and the extent of spontaneous thrombin production vary proportionally, within limits, with the original quantity of thrombin added.

In the fourth experiment (Fig. 4) 6000 units of prothrombin were placed in solution with 500 units of thrombin. No significant change in prothrombin or in thrombin activity was found for a 72 hour period.

### *Activation of Prothrombin with Thromboplastin and Calcium Ions*

It is of considerable theoretical importance to determine whether prothrombin can be activated in the complete absence of Ac-globulin. The prothrombin preparation described above has been shown to be free of this factor on the basis of heat inactivation curves and tests which are very sensitive to Ac-globulin. Thromboplastin, free of Ac-globulin, was prepared as follows by a modification of the sedimentation procedure of Chargaff *et al.* (13). 400 cc. of a crude saline extract of bovine lung (10) were centrifuged at 48,000 R.P.M. in a Sharples supercentrifuge for 30 minutes. The sediment was suspended in 200 cc. of saline and again

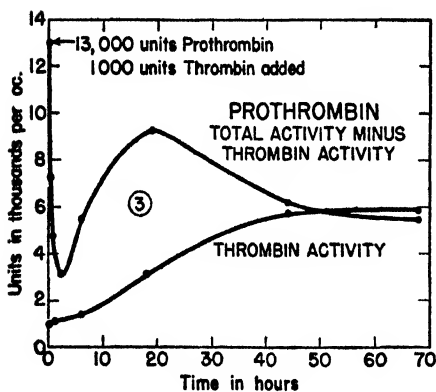


FIG. 3

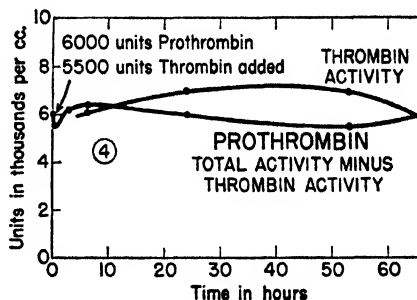


FIG. 4

FIG. 3. Stability of prothrombin at 28° in water solution with 1000 units of thrombin per cc. at pH 7.2.

FIG. 4. Stability of prothrombin at 28° in water solution with 5500 units of thrombin per cc. at pH 7.2.

sedimented by centrifugation at 48,000 R.P.M. for 40 minutes. The sediment was then resuspended in saline to make a total volume of 10 cc.

Our tests showed that this preparation was free of Ac-globulin. This thromboplastin preparation was used to activate various prothrombin solutions at a temperature of 28°. The prothrombin, having been heat-treated as described and dried from aqueous solution, was dissolved in a stock solvent, so that when 1 part of the thromboplastin preparation was mixed with 1 part of stock prothrombin, the resulting solution contained 0.15 per cent calcium chloride (approximately optimum), 0.9 per cent sodium chloride, 5 per cent imidazole buffer by volume, and a thromboplastin concentration approximately 20 times that in crude lung extract. The results are described below and are recorded in Table II.

*Experiment 1*—1 cc. of stock prothrombin solution containing 16,000

TABLE II

*Formation of Thrombin from Prothrombin, Thromboplastin, and Calcium Ions at 38°*

Experiment No.		Time	Pro-thrombin*	Thrombin	Thrombin
			units per cc.	units per cc.	per cent yield
1	Stock thromboplastin	0	8000	0	
		19 min.		520	6.5
		32 "		890	11.1
		1 hr., 10 min.		1550	19.4
		3 hrs., 15 "	1700	3500	44.0
		6 "		3800	47.5
2	Stock thromboplastin, heated	27 "	600	2900	36.0
		0	8000	0	
		14 min.		265	3.3
		27 "		750	9.4
		1 hr.		1380	17.2
		3 hrs.	1800	3000	38.0
3	Stock thromboplastin	6 "		3800	47.5
		27 "	200	2900	36.3
		0	5	0	
		23 min.		Questionable	
		45 "		Trace	
		1 hr., 15 min.		0.5	10.0
4	Stock thromboplastin diluted 1600 times	3 hrs.		1.0	38.0
		6 "	Trace	2.1	42.0
		27 "	"	2.4	48.0
		0	5	0	
		45 min.		Questionable	
		3 hrs.		"	
5	Stock thromboplastin	6 "		"	
		27 "	1	Trace	
		0	300	0	
		20 min.		7	2.3
		30 "		22	7.3
		1 hr., 10 min.		59	19.5
6	Stock thromboplastin diluted 25 times	3 hrs.	90	100	33.3
		5½ "		105	35.0
		22 "	115	105	35.0
		0	300	0	
		20 min.		2	0.67
		35 "		8	2.7
		1 hr., 10 min.		30	10.0
		3 hrs.	135	55	19.3
		5½ "		51	17.0
		22 "	130	70	23.3

\* The prothrombin concentration was measured with Ac-globulin supplied in the two stage assay procedure. Under these circumstances the preformed thrombin was subtracted from the total thrombin yield to give the prothrombin concentration.



units per cc. was mixed with 1 cc. of thromboplastin solution, giving an initial prothrombin concentration of 8000 units per cc. Thrombin was produced slowly. At the end of 3 hours and 15 minutes a thrombin yield of 3500 units per cc. was found. A prothrombin concentration of 1700 units per cc., obtained by analyzing in the presence of an excess of Ac-globulin, indicated that 2800 units per cc. had been destroyed. At 6 hours the thrombin yield reached its highest value of 3800 units per cc. (47.5 per cent yield); this decreased slightly in 27 hours when there remained only 600 units of prothrombin per cc. of solution.

*Experiment 2*—This experiment duplicated Experiment 1 except that the thromboplastin was first heated at 53° for 1½ hours. As compared with Experiment 1, there was no significant change in rate and amount of thrombin formed or in the amount of prothrombin destruction. Since Ac-globulin cannot exist at a temperature of 53°, this experiment is a further indication that the thromboplastin preparation was free of this factor.

*Experiment 3*—In order to determine the effect of concentrated thromboplastin on dilute prothrombin solutions, Experiment 1 was again repeated but this time the initial prothrombin concentration was adjusted to 5 units per cc. of solution. Thrombin production was quite slow. A trace was detected in 45 minutes. This increased to a 10 per cent yield in 1 hour and 15 minutes, and a 48 per cent yield developed during a 27 hour period. Only a trace of prothrombin remained at the 6 and the 27 hour intervals.

*Experiment 4*—This experiment is presented to demonstrate the interaction of the two proteins in dilute solutions. The prothrombin and thromboplastin were mixed in the same proportions as in Experiment 1, but in 1/1600 of the concentration. There was no thrombin production for 6 hours and at 27 hours only a trace was found. Only a part of the prothrombin could be accounted for at that time. This experiment is apparently comparable to the ones described by Owren (11), from which he concluded that thromboplastin and calcium ions cannot activate prothrombin.

*Experiments 5 and 6*—In these two experiments a prothrombin level was selected which is comparable to that in human plasma. In Experiment 5 concentrated thromboplastin was added. Thrombin was produced slowly, reaching a maximum 35 per cent yield in 5½ hours. In Experiment 6 the thromboplastin was diluted 25 times, so that the ratio of prothrombin and thromboplastin was again approximately the same as in Experiment 1. Thrombin production was somewhat slower than in Experiment 5 and the maximum yield (23 per cent) was somewhat less. In both experiments comparatively large amounts of prothrombin re-

mained at the end of 22 hours. However, as in the other experiments there was some destruction of the unconverted prothrombin.

#### DISCUSSION

Seegers, Loomis, and Vandenbelt (4) reported their most active prothrombin preparation to have 15,200 units per mg. of tyrosine. With prothrombin prepared according to their specifications, the amount of Ac-globulin in the preparations was insufficient to give maximum thrombin yields in the two stage prothrombin analysis. Such preparations, giving purity values from 13,000 to 14,000 units per mg. of tyrosine, were considerably more active when analyzed after supplying an excess of Ac-globulin. The specific activity values can be fixed somewhere between 20,000 and 25,000 units per mg. of tyrosine, but the exact activity must await further study. Work now in progress is intended to establish an absolute prothrombin unit on a sound basis. This work deals with stability of dry prothrombin, reference standards, optimum conditions of activation, seasonal variation in plasma prothrombin concentrations, etc.

Mertz, Seegers, and Smith (14) were the first to present evidence which suggested that thrombin destroys prothrombin activity. Owren (11) could obtain only slight evidence for the destruction of prothrombin by thrombin, and Ferguson (15) discounted any such possibility. Experiments reported by these investigators are not necessarily in conflict even though they are inadequate. The data recorded above show that much depends upon the relative quantities of thrombin and prothrombin in solution. Furthermore, the situation is complicated by prothrombin regeneration which is sometimes accompanied by spontaneous formation of thrombin in significant amounts. When 1000 units of thrombin, or less, are added to prothrombin of the strength used above, a fall in prothrombin titer occurs, which is followed by prothrombin regeneration. Spontaneous thrombin production appears to parallel prothrombin regeneration both in amount and in time of appearance. Therefore, it is probable that the regenerated prothrombin forms thrombin with greater ease than purified prothrombin under the conditions of these experiments. When 5500 units of thrombin are added, there is no great change in either prothrombin or thrombin concentrations. Apparently the prothrombin is destroyed and regenerated rapidly; spontaneous thrombin production does not take place to any extent because the reaction is in equilibrium. The generation of 5000 units of thrombin (Fig. 3), in the apparent absence of thromboplastin, Ac-globulin, and calcium ions, is a novelty to this field of research. It is not possible to say definitely that none of these three factors were present. Thromboplastin was certainly not present in appreciable quantities. Ac-globulin was absent from the prothrombin

and could have been present in the thrombin preparation only in traces if at all. Prothrombin and thrombin preparations were both dialyzed against demineralized water to a specific resistance of approximately 3000 ohms. Therefore, the calcium concentration was necessarily extremely low. It seems probable that the correct conditions have been found for the production of thrombin without Ac-globulin, without calcium, and without thromboplastin.

The experiments reported above show that thrombin is produced from prothrombin, thromboplastin, and calcium ions in the complete absence of Ac-globulin. Owren records experiments from which he concluded that thrombin production is not possible from the interaction of prothrombin, thromboplastin, and calcium ions alone (11). His experiments were carried out only at very low prothrombin concentrations for relatively short periods of time. It is not possible to determine from his data what concentrations of thromboplastin were used. If Owren, in addition to low prothrombin concentrations, used weak thromboplastin preparations, it becomes clear from our work why he arrived at his conclusions.

The question may be asked why only a 48 per cent yield of thrombin is obtained when thromboplastin alone is used as the activator. The chief reason appears to be that the reaction is so slow that side reactions produce an effect. One of these, the inactivation of prothrombin by thrombin, is described in this paper. Even in the presence of Ac-globulin, a maximum thrombin yield is not obtained unless an optimum amount of the accelerator is present (7, 8). Decreased thrombin yields apparently result when any factor slows the rate of interaction of prothrombin and thromboplastin. This was pointed out in 1944 (16) from studies which involved the effects of alterations in pH, calcium, electrolyte concentration, etc., on prothrombin activation.

On the basis of experiments which indicated that Ac-globulin catalyzes the reaction between prothrombin, thromboplastin, and calcium ions, it was decided to call this factor an accelerator (7). Since it was also found to be a globulin, the name accelerator globulin or Ac-globulin was proposed (7). The data reported here demonstrate that thrombin is produced in the absence of Ac-globulin. The latter speeds up the reaction (6-8). Therefore it is rightly designated as an accelerator and cannot be considered as a component of prothrombin.

#### SUMMARY

An extensive study of previously reported methods for the preparation of prothrombin has been made. Modifications are introduced which consistently give highly active preparations, stable in solution for more than 24 hours at room temperature. The products contain approximately

0.6 per cent Ac-globulin by weight. Efforts to remove this last quantity of Ac-globulin with fractionation procedures have repeatedly failed. However, the prothrombin is freed entirely of this activity by heating at 53° in aqueous solution. Heating in saline solution results in losses of both prothrombin and Ac-globulin.

Extremely small amounts of thrombin destroy prothrombin. Larger amounts of thrombin produce a diphasic effect upon prothrombin; there is first a destruction, followed by a regeneration of prothrombin activity. The regenerated prothrombin appears to be changed in such a way that it may form thrombin in the absence of thromboplastin, Ac-globulin, and calcium ions.

Thromboplastin and calcium ions activate prothrombin in the absence of Ac-globulin, but the reaction is slow and the final yield of thrombin is only about half of that produced in the presence of an optimum amount of Ac-globulin.

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APPARENT DISSOCIATION EXPONENTS OF QUININE,  
PAMAQUINE, AND A QUINOLYLPIPERIDYL-  
CARBINOL; APPLICATION OF AN  
EXTENDED pH SCALE\*

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Apparent dissociation exponents for quinine and pamaquine (plasmochin) have been determined potentiometrically by Christophers (2) and by Christophers and Fulton (3). Recently Elderfield, Craig, *et al.* (4) have demonstrated by application of Craig's counter-current distribution technique (5, 6) that various commercial samples of pamaquine are inhomogeneous and contain isomers of pamaquine to the extent of 12 to 30 per cent of the total material.

With a sample of pure pamaquine (6-methoxy-8-(4'-diethylamino-1'-methylbutylamino)-quinoline), obtained through the courtesy of Dr. Craig, we have determined the apparent dissociation exponents of this compound by potentiometric and spectrophotometric methods. Also, a hitherto undescribed reversible proton exchange involving pamaquine in rather concentrated aqueous solutions of sulfuric and hydrochloric acids has been studied by spectrophotometry. This proton exchange of pamaquine and a similar one for the structural isomer of pamaquine, SN-3294,<sup>1</sup> have been evaluated in terms of the extended pH scale of Michaelis. In addition, the exponents for quinine and an Ainley-King carbinol (7), SN-2157,<sup>1</sup> have been evaluated by potentiometric and spectrophotometric methods.

*Formulation of Equilibria*—By potentiometric titration of aqueous solutions of quinine, pamaquine, and SN-2157 two widely separated dissociation exponents,  $pK'_1$  and  $pK'_2$ , can be evaluated for each compound. In view of the considerable difference in magnitude of  $pK'_2$  and  $pK'_1$  for each of

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<sup>1</sup> Numbers preceded by the letters SN are the code numbers assigned by the Office of the Survey of Antimalarial Drugs. Data concerning these and many other compounds have been published (8). SN-3294 is 6-methoxy-4-(4'-diethylamino-1'-methylbutylamino)-quinoline. SN-2157 is 4-(6-methoxyquinolyl)- $\alpha$ -piperidylcarbinol.

these compounds, it can be assumed that these exponents are practically identical with *intrinsic* group exponents as defined by Adams (9); *i.e.*, each of these exponents refers to the proton exchange involving a single, particular proton acceptor in the molecule. An exponent,<sup>2</sup>  $pK'$ , can be evaluated spectrophotometrically (Figs. 1 and 2) for each of these compounds by a procedure similar to the one described in a study of various 4-aminoquinolines (10). This exponent is concerned with a proton exchange involving an acceptor associated with the aromatic nucleus. The value of  $pK'$ , was found to be practically identical with the value of  $pK'_1$ ,

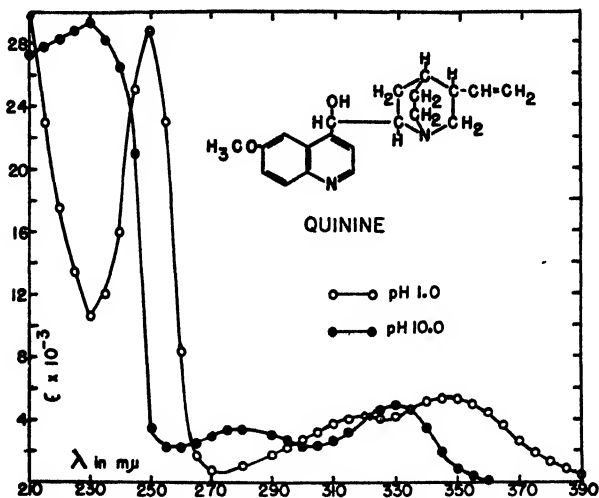
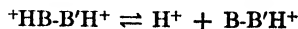


Fig. 1. Spectrophotometric absorption curves for quinine in dilute aqueous solutions. Identical curves were obtained for SN-2157 at these values of pH.

in each case; therefore both  $pK'_1$  and  $pK'$  must refer to the same acceptor in the aromatic nucleus. This acceptor probably is the nitrogen atom of the quinoline ring. The equilibrium evaluated by  $pK'_1$  and  $pK'$ , can be represented as follows



in which B represents a proton acceptor associated with the aromatic nucleus and B' represents the proton acceptor of the aliphatic side chain. At constant ionic strength,

<sup>2</sup>  $pK' = pH - \log [B \cdot B'H^+]/[{}^+HB \cdot B'H^+] = pH - \log [(\epsilon_{B'H^+} - \epsilon)/(\epsilon - \epsilon_B)]$ , in which B represents a proton acceptor associated with the aromatic nucleus and B' corresponds to the proton acceptor of the aliphatic side chain.  $\epsilon_{B'H^+}$  and  $\epsilon_B$  are, respectively, the molecular absorption coefficients of the proton donor and proton acceptor species of the aromatic nucleus.  $\epsilon$  is the molecular absorption coefficient at a pH value at which both species are present.

$$pK'_1 = pK'_2 = pH - \log \frac{[B-B'H^+]}{[+HB-B'H^+]} \quad (1)$$

in which brackets are used to designate concentrations.

Inasmuch as  $pK'_1$  refers to a proton exchange involving an acceptor associated with the aromatic nucleus,  $pK'_2$ , which cannot be evaluated spectrophotometrically in the spectral range studied, must be concerned

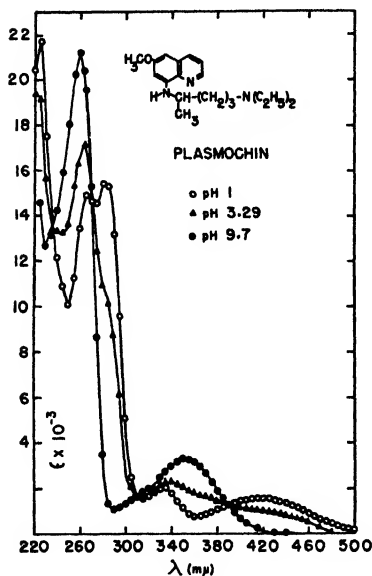


FIG. 2

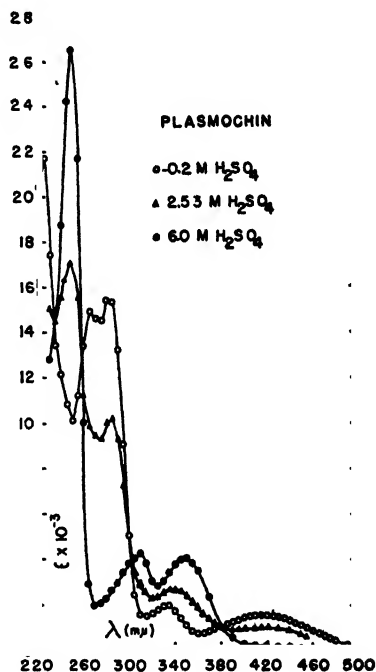


FIG. 3

FIGS. 2 AND 3. Spectrophotometric absorption curves for pamaquine in dilute aqueous solutions (Fig. 2) and in aqueous solutions of sulfuric acid (Fig. 3).

with a proton exchange involving an acceptor in the aliphatic side chain. This acceptor is the quinuclidine nitrogen of quinine, the piperidyl nitrogen of SN-2157, and the diethylamino nitrogen of pamaquine. The equilibrium can be represented as follows:



At constant ionic strength,  $pK'_2$  is defined by equation (2).

$$pK'_2 = pH - \log \frac{[B-B']}{[B-B'H^+]} \quad (2)$$

In aqueous solutions of sulfuric acid from 0.2 to 7 M, another reversible transformation of pamaquine is observed spectrophotometrically (Fig. 3).



It seems highly probable that this transformation is concerned with another proton exchange involving the aromatic nucleus, the secondary amino nitrogen at position 8 probably being the proton acceptor. In Fig. 3 the absorption curve for pamaquine in 0.2 M sulfuric acid is ascribed to the monopolar cationic species of the aromatic nucleus,<sup>3</sup> the proton probably being attached to the ring nitrogen. The curve in 6 M sulfuric acid is ascribed to the dipolar cationic species of the aromatic nucleus, 1 proton being attached to the ring nitrogen and the other to the 8-amino nitrogen. The transformation is practically complete in 6 M sulfuric acid and is entirely complete in 7 M. The curve for pamaquine in 2.53 M sulfuric acid is ascribed to an intermediate equilibrium state in which both species are present. The transformation can be reversed simply by decreasing the concentration of sulfuric acid by dilution of the solution with water. This second proton exchange involving the aromatic nucleus of pamaquine is comparable to that of the 4-aminoquinolines described previously (10). This proton exchange involving pamaquine also occurs in concentrated aqueous solutions of hydrochloric acid.

It was desirable to evaluate this second proton exchange of pamaquine in terms of an extended pH scale. A generalized basis for the extension and alternative formulation of a scale for proton exchanges has been presented by Clark (11), and two experimental extensions of the scale have been described (12-18). Hammett *et al.* (12-17) have defined an "acidity function,"  $H_0$ , and have evaluated a scale for this function in concentrated aqueous solutions of sulfuric acid by color comparison and spectrophotometry with a series of indicators of the same charge type and of graded and overlapping degrees of proton-accepting strength. The acidity function is defined by equations (3) and (4)

$$H_0 = pK + \log \frac{[B]}{[BH^+]} \quad (3)$$

$$H_0 = -\log (H^+) \frac{f_B}{f_{BH^+}} \quad (4)$$

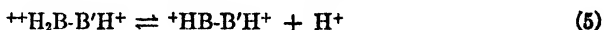
in which  $pK$  is the true dissociation exponent of an indicator which enters into the proton equilibrium,  $BH^+ \rightleftharpoons B + H^+$ . In equation (4) parentheses represent activity and  $f_B$  and  $f_{BH^+}$  are activity coefficients. The acidity function scale is based upon the fundamental assumption that the ratio  $f_B:f_{BH^+}$  in a given solution is the same for all proton acceptor-donors of the same charge type. Hall and Spengeman (19) have recalculated the

\* The molecule as a whole is a dipolar cation in 0.2 M sulfuric acid and a tripolar cation in 7 M, inasmuch as a proton is attached to the nitrogen of the aliphatic side chain throughout this range.

data of Hammett *et al.* and have found that in aqueous solutions of sulfuric acid from 1 to 18 M  $H_0$  is a linear function of the molarity of sulfuric acid, with a slope of approximately 0.5.

Michaelis and Granick (18) have described an extension of the pH scale which is based upon the relationship between pH and the *differences* between the normal potentials of the two steps in the oxidation-reduction of various thiazines and the extrapolation of this relationship to data obtained with these indicators in concentrated aqueous solutions of sulfuric acid. The procedure required orientation of the extrapolation by spectrophotometric determinations of the mid-points of two of the proton exchanges involved. The values of pH thus determined were found to be a linear function of the molarity of the sulfuric acid, above 1 M, with a slope of 0.57.

The proton exchanges involving pamaquine and SN-3294 in aqueous solutions of sulfuric acid can be represented by equation (5)



in which B refers to the aromatic nucleus with two proton acceptors (ring nitrogen and secondary amino nitrogen) and B' represents the proton acceptor of the side chain. The dissociation exponent is defined by equation (6).

$$pK_{(H_2SO_4)} = pH \text{ (or } H_0) - \log \frac{[{}^+HB \cdot B'H^+]}{[{}^{++}H_2B \cdot B'H^+]} \quad (6)$$

It should be noted that the  $H_0$  scale of Hammett and Deyrup (12) was evaluated by means of a series of indicators of simpler charge type than that represented in equation (5). The importance of this difference in charge type will be emphasized later.

#### EXPERIMENTAL

Values of  $pK'_1$ ,  $pK'_2$ , and  $pK'$ , were determined essentially as described in the preceding paper on 4-aminoquinolines (10). For the determination of  $pK_{(H_2SO_4)}$  for pamaquine and SN-3294 (equation (6)), optical densities of a series of solutions of the compound in various concentrations of  $H_2SO_4$  were determined with a Beckman photoelectric quartz spectrophotometer, model DU, with 1 cm. fused silica cuvettes. The nominal spectral interval isolated, evaluated from data for dispersion and slit widths furnished with the Beckman instrument, was maintained at  $2 \mu$ . Sulfuric acid solutions of concentrations identical with the corresponding principal solutions were used as blanks. Absorption data were calculated as molecular absorption coefficients,  $\epsilon$ , which are defined by the equation,  $-\log T = D = \epsilon cl$ , in which  $T$  is the transmittancy expressed as a fraction of unity,  $D$  is the

optical density,  $c$  is the concentration of the compound in moles per liter, and  $l$  is the length, in cm., of the light path through the solution. The concentrations of sulfuric acid in the series of solutions were determined by titration with standard 1 M sodium hydroxide, and the concentrations were checked in some cases by measurement of density for comparison with data in the "International critical tables." Pipettes used for measuring samples of the viscous solutions of sulfuric acid were specially calibrated "to contain" and were rinsed with distilled water to insure complete and accurate

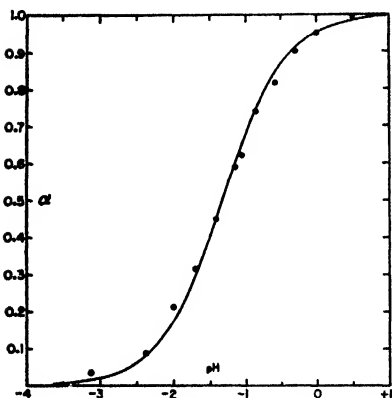


FIG. 4

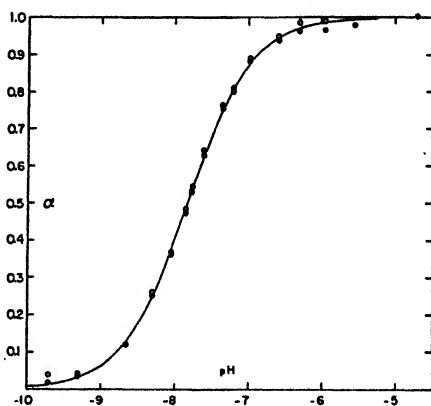


FIG. 5

FIGS. 4 AND 5. Test of the relationship,  $\text{pH} = \text{pK}_{(\text{H}_2\text{SO}_4)} + \log [\alpha/(1 - \alpha)]$ , for pamaquine (Fig. 4) and SN-3294 (Fig. 5). pH is the extended scale of Michaelis calculated from the molar concentrations of sulfuric acid. The theoretical curves are drawn for the following values of  $\text{pK}_{(\text{H}_2\text{SO}_4)}$ : for pamaquine,  $-1.33$ ; for SN-3294,  $-7.83$ . In the case of pamaquine, the symbols represent the average experimental values of  $\alpha$  calculated from optical densities measured at wave-lengths 420, 360, 350, 310, 280, and 270  $\text{m}\mu$  (Fig. 3). In the case of SN-3294, O represents the experimental values of  $\alpha$  calculated from optical densities at 375  $\text{m}\mu$ , and ● the values calculated from densities at 351  $\text{m}\mu$  (see the absorption curves in the previous paper (10)).

transfer. Values of pH or  $H_0$  were calculated from the molar concentrations of sulfuric acid by means of the scale of Michaelis (18) or the scale of Hammett (12) as recalculated by Hall and Spengeman (19). For evaluation of  $\text{pK}_{(\text{H}_2\text{SO}_4)}$ , the data for pH and the corresponding absorption coefficients at some selected wave-length were rectified by the general method of Reed and Berkson (20) with the adaptation of Clark *et al.* (21), applied essentially as described previously (10) for the calculation of  $\text{pK}'$ . Corrections for "medium effects," as defined by Flexser, Hammett, and Dingwall (16), were made essentially as described by these authors. In Figs. 4 and 5, data for these compounds are presented in terms of the extended pH scale of Michaelis and of  $\alpha$ , the degree of dissociation of the proton

donor,  ${}^{++}\text{H}_2\text{B-B'H}^+$ , into  ${}^+\text{HB-B'H}^+$  and  $\text{H}^+$  calculated from the absorption coefficients. The curves are drawn to the theoretical relationship,  $\text{pH} = \text{pK}_{(\text{H}_2\text{SO}_4)} + \log [\alpha/(1 - \alpha)]$ .

In preparing concentrated aqueous solutions of hydrochloric acid for study of the second proton exchange involving the aromatic nucleus of pamaquine in this solvent, it was found to be essential to use freshly opened bottles of C.P. concentrated HCl. Partially empty bottles which had been exposed to air and sunlight apparently contained traces of free chlorine which chlorinated (or oxidized) pamaquine with accompanying changes in spectrophotometric absorption which were irreversible by dilution with water.

TABLE I  
*Dissociation Exponents (30°) of Various Derivatives of Quinoline*

Compound*	Ionic strength, 0.1			$\text{pK}_{(\text{H}_2\text{SO}_4)}$ (scale of Michaelis)
	$\text{pK}'_0$	$\text{pK}'_1$	$\text{pK}'_2$	
Quinine .....	4.33	4.32	8.4	
SN-2157 .....	4.34	4.36	9.3	
Pamaquine .....	3.49	3.48	10.2	-1.33†
SN-3294 .....	8.73	8.68	10.2	-7.83‡

\* SN-2157 is 4-(6-methoxyquinolyl)- $\alpha$ -piperidylcarbinol, and SN-3294 is 6-methoxy-4-(4'-diethylamino-1'-methylbutylamino)-quinoline.

† Mid-point, 2.86 M sulfuric acid or 3.89 M hydrochloric acid.

‡ Mid-point, 14.26 M sulfuric acid.

#### DISCUSSION

Apparent dissociation exponents (ionic strength 0.1; 30°) for the proton exchanges occurring in dilute aqueous solutions are compiled in Table I. Our values for  $\text{pK}'_1$  and  $\text{pK}'_2$  for quinine are in fair agreement with those obtained potentiometrically by Christophers (2) and by Kolthoff (22), although the conditions of the measurements were not exactly comparable.<sup>4</sup> Christophers reported a value of 4.21 for  $\text{pK}'_1$  and 8.36 for  $\text{pK}'_2$  at 20°. The ionic strength was not specified explicitly, and apparently it was not kept constant throughout the titrations, but, on the basis of the concentrations of the solutions titrated, the ionic strength at the mid-points was 0.02 in evaluating  $\text{pK}'_1$  and 0.002 in evaluating  $\text{pK}'_2$ . Kolthoff's values for  $\text{pK}'_1$  and  $\text{pK}'_2$  of quinine were 4.50 and 8.23, respectively, at 15°. Our value for  $\text{pK}'_0$ , the spectrophotometrically determined dissociation ex-

<sup>4</sup> Values reported by these investigators were expressed in terms of "basic" dissociation exponents. The values quoted above have been recalculated in terms of proton dissociation exponents as defined in the present paper.

ponent for the proton exchange concerned with the aromatic nucleus of quinine, is practically identical with the corresponding value of  $pK'_1$  determined potentiometrically (Table I). The proton acceptor involved in the equilibrium evaluated by  $pK'_1$  ( $= pK'_1$ ) undoubtedly is the nitrogen atom of the quinoline ring. The proton acceptor in quinine which is concerned in the proton exchange evaluated potentiometrically as  $pK'_2$  is the quinuclidine nitrogen atom.

The values of  $pK'_1$  and  $pK'_2$  for SN-2157 (Table I) agree closely with the corresponding exponents for quinine, and again the proton acceptor concerned is the quinoline ring nitrogen. Spectrophotometric absorption curves for the proton donor and proton acceptor species of quinine (Fig. 1) and SN-2157 are identical in the spectral range studied. The piperidyl nitrogen of SN-2157 is a stronger proton acceptor than the quinuclidine nitrogen of quinine ( $pK'_2$ , 9.3 and 8.4, respectively). A dissociation exponent of 10.98 has been reported for 2-methylpiperidine (23). By contrast, the value of  $pK'_2$  for SN-2157 reflects the electron-attracting effects of the carbinol group and the quinoline ring.

The values reported by Christophers and Fulton<sup>4</sup> (3) for the apparent dissociation exponents of pamaquine were 3.55 for  $pK'_1$  and 10.13 for  $pK'_2$  at 20°, and the ionic strength at the mid-points apparently was about 0.009 in evaluating  $pK'_1$  and 0.0002 in determining  $pK'_2$ . Our potentiometric values (Table I) are in reasonable agreement with those of Christophers and Fulton, and again the exponent determined spectrophotometrically,  $pK'_2$ , is practically identical with  $pK'_1$ . The proton exchange evaluated by  $pK'_2$  involves the diethylamino nitrogen of the side chain of pamaquine as acceptor. In the case of pamaquine, the proton acceptor corresponding to  $pK'_1$  ( $= pK'_1$ ) is somewhat uncertain, inasmuch as both the nitrogen of the quinoline ring and the 8-amino nitrogen are possible acceptors. The acceptor principally involved should be the one possessing the greatest relative proton-accepting strength. An approximate estimate of the relative intrinsic strengths of these acceptors can be obtained by comparing data for quinoline and for an N-alkylaniline. The dissociation exponents for quinoline and for N-*n*-propylaniline are 4.94 and 5.02, respectively (24, 23). Mutual weakening of the strengths of these acceptors might be expected through inductive action. Resonance effects in the entire proton acceptor molecule which might tend to strengthen proton acceptance probably would be less important than the mutual inductive effect (operating in the opposite direction), but any resonance effect of this type probably would tend to "strengthen" the ring nitrogen more than the 8-amino nitrogen. Both of these acceptors would be weakened by the electrostatic and inductive effects of the diethylamino group of the side chain which would bear the positive charge of an attached proton in the range of pH in which the

proton exchanges involving the aromatic nucleus are evaluated. However, this latter effect should weaken the 8-amino nitrogen more than the ring nitrogen, inasmuch as the former is closer than the latter to the diethyl-amino group. Furthermore, the methoxy group, the effect of which is principally inductive when meta-substituted, would be expected to have a greater weakening effect on the proton-accepting strength of the 8-amino nitrogen than of the ring nitrogen. Thus, the ring nitrogen would be anticipated to be the stronger proton acceptor. Nevertheless, there would appear to be a statistical probability that *both* acceptors would be involved to some extent in the range of pH in which  $pK'$ , was evaluated. However, isosbestic points<sup>5</sup> were maintained throughout the range of pH in which the change in spectrophotometric absorption occurred (Fig. 3). Maintenance of such isosbestic points generally is accepted as evidence that only two absorbing species are involved in the change. For this reason and the fact that a simple one proton relationship was followed, we believe that the proton exchange for which  $pK'$ , ( $= pK'_1$ ) is a measure involves the ring nitrogen of pamaquine almost exclusively. This conclusion is in disagreement with that of Christophers and Fulton (3) who decided, without stating their reasons, that the 8-amino group was involved.

If the proton exchange evaluated by  $pK'$ , ( $= pK'_1$ ) involves the ring nitrogen of pamaquine, then the proton exchange evaluated from 0.2 to 7 M sulfuric acid (Figs. 3 and 4) or from 0.4 to 8 M hydrochloric acid must involve the 8-amino group as acceptor. The dissociation exponent on the extended scale of Michaelis is  $-1.33$  for aqueous solutions of sulfuric acid (Fig. 4 and Table I). The mid-point of the change in aqueous solutions of hydrochloric acid is 3.89 M. The spectrophotometric absorption curves for pamaquine at the extremes of the transformation in HCl are identical with corresponding curves in  $H_2SO_4$ . In summary, although the *intrinsic* strengths of the quinoline ring nitrogen and the secondary 8-amino group are approximately the same, the latter group is weakened relative to the former by diethylamino and methoxy groups and the difference is further accentuated after the ring nitrogen has accepted a proton by reason of the electrostatic and inductive effects of this positive charge.

Data for the 4-aminoquinoline derivative, SN-3294, are included in Table I for contrast with corresponding data for its structural isomer, pamaquine. Data for SN-3294 were reported previously (10), but no attempt was made there to evaluate the proton exchange in concentrated solutions of sulfuric acid in terms of the extended pH scale of Michaelis as reported in the present paper (Fig. 5). The marked strengthening of the acceptance of the first proton by the aromatic nucleus of SN-3294 and the

<sup>5</sup> The significance of the isosbestic point is discussed by Clark ((11) pp. 152-154).

great weakening of the acceptance of the second proton, as contrasted with corresponding proton exchanges of pamaquine, can be attributed to the powerful resonance of the monopolar cation of the aromatic nucleus of SN-3294. The resonance hybrid of this species probably receives contributions both from Kekulé and quinonoid structures, as first suggested for the parent 4-aminoquinoline by Albert and Goldacre (24) and as emphasized for a series of derivatives of this compound in a previous paper (10).

The spectrophotometric absorption data for pamaquine and for SN-3294 in aqueous solutions of sulfuric acid were formulated both in terms of the acidity function scale of Hammett and the extended pH scale of Michaelis. Conformity of the data to the theoretical relationship,  $\text{pH}$  (or  $\text{H}_0$ ) =  $\text{pK}_{(\text{H}_2\text{SO}_4)} + \log [\alpha/(1 - \alpha)]$ , was found to be better when the pH scale of Michaelis (Figs. 4 and 5) was used rather than the acidity function scale of Hammett. It seems likely that this can be attributed largely to the fact that these proton exchanges of pamaquine and SN-3294 are of a charge type which corresponds more closely to that of the thiazines employed by Michaelis (although the charge type varied within his series) than of the series of indicators of Hammett and Deyrup (12). It was emphasized clearly by Hammett and Deyrup that several acidity functions ( $\text{H}_-$ ,  $\text{H}_0$ ,  $\text{H}_+$ ,  $\text{H}_{++}$ , etc.) would be expected on the basis of the charge types of the indicators.

In addition to the proton exchanges discussed above, quinine and pamaquine exhibit reversible changes in spectrophotometric absorption and fluorescence in 8 to 18 M sulfuric acid which are due either to proton exchanges involving the methoxy groups as acceptors or to "medium effects." These changes will be described in the succeeding paper on a fluorometric method for the determination of these compounds in biological samples.

#### SUMMARY

Apparent dissociation exponents for proton exchanges involving the aromatic nuclei (ring nitrogen atoms as probable acceptors) of quinine, pamaquine, and 4-(6-methoxyquinolyl)- $\alpha$ -piperidylcarbinol have been determined potentiometrically and spectrophotometrically, and the average values are 4.33, 3.49, and 4.35, respectively. In contrast, the corresponding exponent for SN-3294, a structural isomer of pamaquine with the secondary amino group in position 4 instead of position 8, is 8.71. Apparent dissociation exponents determined potentiometrically for the proton exchanges involving the side chains of these compounds are 8.4, 10.2, and 9.3, respectively.

In concentrated aqueous solutions of sulfuric acid, second proton exchanges involving the aromatic nuclei (secondary amino groups as probable

acceptors) of pamaquine and SN-3294 have been evaluated spectrophotometrically, and the equilibria have been formulated in terms of the extended pH scale of Michaelis, the values of the dissociation exponents being  $-1.33$  and  $-7.83$ , respectively. The marked differences in the proton-accepting strengths of pamaquine and SN-3294 are attributed to the special resonance of the latter.

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# A FLUOROMETRIC METHOD FOR THE DETERMINATION OF PAMAQUINE, SN-13276, AND SN-3294\*

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Brodie *et al.* (1) have described a photometric method for the determination of pamaquine<sup>1</sup> which is based upon the coupling of this compound with diazotized sulfanilic acid. This procedure is satisfactory, but alternative methods are desirable, particularly for studies of the disposition of this antimalarial compound in the tissues and body fluids of various animals. Alternative analytical methods, based upon different properties of a therapeutic compound, provide information concerning the degradation and subsequent disposal of the drug which a single method cannot yield so readily. The fluorescence of pamaquine, SN-13276, and SN-3294<sup>1</sup> in concentrated sulfuric acid provides the basis for the analytical method described in the present paper.

## Analytical Procedure

### Reagents—

1. Stock standard solution of pamaquine, 100 mg. per liter. Dissolve 161 mg. of the citrate salt<sup>2</sup> in 0.1 N sulfuric acid, and dilute the solution to 1 liter with the acid. This solution is stable for several weeks when stored in a refrigerator. Working standards are prepared daily by dilution with 0.1 N H<sub>2</sub>SO<sub>4</sub>.

2. 0.1 N NaOH.

3. 1.0 N NaOH.

4. Hexane. Eastman Kodak Company, practical grade (from petroleum). This crude solvent contains both volatile and non-volatile substances which fluoresce in concentrated sulfuric acid. The solvent is

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<sup>1</sup> Pamaquine (plasmochin) is 6-methoxy-8-(4'-diethylamino-1'-methylbutylamino)-quinoline. Numbers preceded by the letters, SN, are the code numbers assigned by the Office of the Survey of Antimalarial Drugs. SN-13276 is 6-methoxy-8-(5'-isopropylaminoamylamino)-quinoline. SN-3294 is 6-methoxy-4-(4'-diethyl-amino-1'-methylbutylamino)-quinoline.

<sup>2</sup> We are indebted to Dr. Leslie Hellerman and Dr. Curt C. Porter for a sample of purified pamaquine citrate prepared, by their unpublished procedure, from a commercial sample of pamaquine.

freed of the non-volatile substances by distillation in an all-glass apparatus at atmospheric pressure. Collect the fraction boiling from 66–72° and store it in a glass-stoppered bottle. The volatile, fluorescent substances do not interfere in the method described below, since the final hexane extract is evaporated prior to addition of sulfuric acid.

5. *n*-Butyl alcohol, reagent grade.

6. 0.1 N HCl.

7. Sulfuric acid, c.p., analytical reagent grade, specific gravity 1.84. Most samples of concentrated sulfuric acid exhibit some fluorescence when irradiated by means of a mercury vapor lamp. However, "blank" fluorescence from this source is quite constant for a particular batch of the acid, and corrections are readily made in the calculations.

*Procedure*—In the following procedure pamaquine is specified, but the same directions apply in the determination of SN-13276.

Add 1 to 10 ml. of plasma or other biological sample<sup>3</sup> (containing 0.2 to 5  $\gamma$  of pamaquine) and an equal volume of 0.1 N NaOH to 30 ml. of redistilled hexane in a glass-stoppered bottle. Shake the mixture for 15 minutes on a mechanical shaker. Centrifuge to separate the phases as completely as possible. If a thick layer of emulsion exists between the phases after the first centrifugation, break the emulsion with a stirring rod and repeat the centrifugation. Add 0.5 ml. of *n*-butyl alcohol<sup>4</sup> to the hexane phase with minimum disturbance of the aqueous layer. With a pipette transfer exactly 25 ml. (or, if necessary, a smaller volume accurately measured) of the hexane phase to a small, tapered separatory funnel; add 5 ml. of 0.1 N HCl and shake vigorously for several minutes. Allow the phases to separate; then transfer the entire acid aqueous phase to another separatory funnel. Extract the hexane again with 2 ml. of 0.1 N HCl and combine this second acid extract with the first. Introduce 25 ml. of redistilled hexane into the funnel containing the acid extracts. Add 1 ml. of 1.0 N NaOH and shake the mixture vigorously for 5 minutes. Allow the phases to separate and add 0.5 ml. of *n*-butyl alcohol to the hexane with minimum disturbance of the aqueous layer. Draw off the aqueous phase and discard. Transfer the hexane quantitatively to a 100 ml. distilling flask and evaporate to dryness by distillation under diminished pressure (water aspirator pump) in an all-glass still. Maintain the water bath in which the distilling flask is immersed at 30–50° during the distillation. It is important to remove all traces of the solvent from the flask. Then place exactly 8 ml. (or slightly more than the minimum volume required

<sup>3</sup> Samples of organ tissues, feces, etc., can be prepared for analysis by the procedure described by Brodie *et al.* (2).

<sup>4</sup> Butyl alcohol is added at this point in order to minimize loss of pamaquine by adsorption on the walls of the glass vessel (1, 2).

for the particular fluorometer employed) of concentrated sulfuric acid in the distilling flask and spread the acid over the entire inner surface of the flask in order to bring all of the pamaquine into solution. Transfer the acid to a cuvette and determine the fluorescence in a fluorometer.<sup>5</sup> The irradiation of the sample is best carried out by means of a mercury vapor lamp. For the greatest sensitivity, Corning Filter 5970 (new code number of the Corning Glass Works), specially ground to a thickness of 1 mm., should be placed between the lamp and the cuvette. However, Coleman Filter B<sub>1</sub>S also can be used in this "primary" position with a small decrease in analytical sensitivity. The secondary filter inserted between the cuvette and the photocell should be Corning Filter 3060 or 3389 or Coleman Filter PC<sub>1</sub>. Filter 3060 will provide greatest analytical sensitivity, but with fluorometers in which a considerable amount of the primary radiation reaches the photocell by reflection from the sides of the cuvettes or by other sources of "scattering," it may be necessary to use Filter 3389 instead of Filter 3060 to minimize the blank from this source. Also, when blanks due to scattered radiation are serious, it may be necessary to use a combination of Filters 3389 and 5030 in the secondary position. Filter 5030 blocks the radiation in the "red" range of the spectrum which also is transmitted by primary Filter 5970. Transmittance-wave-length curves for these filters are presented in "Glass color filters," a publication of the Corning Glass Works.

Standards should be carried through the entire procedure with each set of unknowns, and the concentrations of the latter are calculated from the fluorometric values of these standards. A reagent blank, which includes the blank due to the sulfuric acid, should be determined by substituting distilled water for the biological sample in the procedure. In the calculations, the fluorometric value of the reagent blank is deducted from the values for standards and unknowns. The intensity of fluorescence of the standards is directly proportional to the concentration of pamaquine.

The same procedure can be used for the determination of SN-3294 in amounts as small as 0.1  $\gamma$ , but analytical recoveries are more nearly quantitative if freshly redistilled ethylene dichloride (1,2-dichloroethane) is used in place of hexane for the extraction of this compound.

#### RESULTS AND DISCUSSION

It is shown in Table I that pamaquine and SN-3294 added to plasma are recoverable with adequate precision by this analytical procedure. The method is equally applicable to the determination of SN-13276. The amount of interfering fluorescent material in normal plasma is negligible when the analysis is carried out by the double extraction procedure de-

<sup>5</sup> Coleman photofluorometer, or Klett fluorometer, or other similar instrument.

scribed above. Attempts to shorten the method by extracting pamaquine directly from the initial organic solvent extract with concentrated sulfuric acid, or by evaporating the *initial* extract and adding sulfuric acid, were unsatisfactory owing to excessive "blank" fluorescence derived both from the solvent and from normal plasma under these conditions. When the double extraction procedure is used as described, the reagent blank is not negligible but is quite constant and reproducible with a particular batch of reagents. In order to minimize the blank, it is particularly important to distil the solvents prior to use.

An attempt was made to determine the basis for the fluorescence of pamaquine and SN-3294 in concentrated sulfuric acid. As presented in

TABLE I

*Analytical Recovery of Pamaquine and SN-3294 from Plasma*

Data for recoveries are the averages of five determinations at each concentration.

Compound	Concentration of compound in plasma (theoretical)	Volume of plasma sample analyzed	Average concentration of compound in plasma (found)	Average recovery	Average deviation of a single determination from average
	$\gamma$ per l.	ml.	$\gamma$ per l.	per cent	per cent
Pamaquine	20	10	18.4	92.0	$\pm 10.5$
	50	5	47.2	94.4	$\pm 8.4$
	100	5	95.2	95.2	$\pm 6.2$
	200	5	195.0	97.5	$\pm 5.0$
	500	2	490.0	98.0	$\pm 4.8$
SN-3294	10	10	8.9	89.0	$\pm 12.0$
	20	10	18.7	93.5	$\pm 9.3$
	50	5	48.1	96.2	$\pm 7.0$
	100	5	95.3	95.3	$\pm 5.5$

the preceding paper (3), spectrophotometric and potentiometric evidence demonstrated that in 7 M sulfuric acid pamaquine is a tripolar cation with protons attached to the diethylamino nitrogen of the side chain, the quinoline ring nitrogen, and the secondary 8-amino nitrogen. When the concentration of sulfuric acid is increased from 8 to 18 M, there is a small shift in the absorption spectrum of pamaquine (Fig. 1). Solutions of pamaquine in 7 M sulfuric acid are practically non-fluorescent when irradiated by sunlight or by the 365  $m\mu$  line of the mercury emission spectrum. As the concentration of sulfuric acid is increased from 8 to 18 M, there is a progressive increase in the fluorescence, solutions of pamaquine in 18 M sulfuric acid exhibiting brilliant violet-blue fluorescence. The mid-point of the change is in 13.76 M sulfuric acid. These changes in spectrophotometric absorption and fluorescence might be attributed to "medium effects," i.e. to

progressive changes in properties of the medium such as density and viscosity, but there would seem to be a possibility that the changes are due, at least in part, to a proton exchange involving the methoxy group of pamaquine as proton acceptor. The fact that the changes in absorption and fluorescence can be reversed by dilution of the concentrated acid solutions with water is in favor of the interpretation of the changes as due to a reversible proton exchange. In favor of the conclusion that the methoxy group is involved is the fact that desmethoxy pamaquine (8-(4'-diethyl-

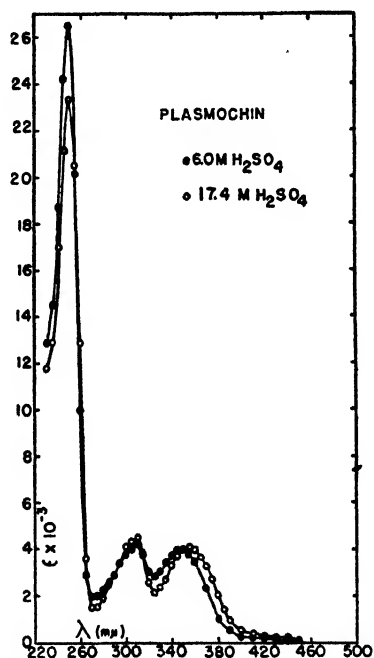


FIG. 1. Change in spectrophotometric absorption by pamaquine in aqueous sulfuric acid from 6 to 17.4 M.

amino-1'-methylbutylamino)-quinoline) is practically non-fluorescent in both 8 M and 18 M sulfuric acid.

Inasmuch as quinine also is a derivative of 6-methoxyquinoline, it was desirable to study the spectrophotometric absorption and fluorescence of this compound in concentrated aqueous solutions of sulfuric acid for comparison with pamaquine. As presented in the preceding paper (3), spectrophotometric and potentiometric evidence demonstrated that in 0.1 M sulfuric acid quinine is a dipolar cation with protons attached to the quinuclidine nitrogen and the nitrogen of the quinoline ring. The absorption spectrum of quinine changes progressively as the concentration of sul-

furic acid is increased from 0.1 to 17.5 M. The complete absorption spectra of quinine for the extremes of this range of concentrations of  $\text{H}_2\text{SO}_4$  are shown in Fig. 2, and details of the progressive changes in a selected region

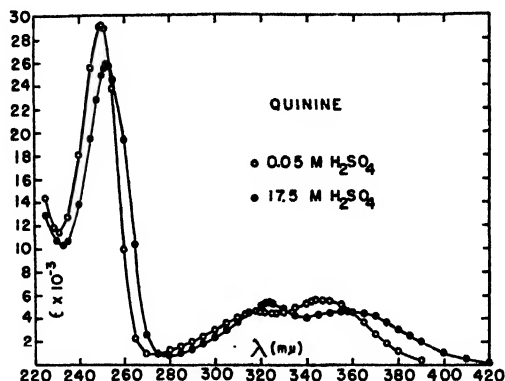


FIG. 2. Spectrophotometric absorption curves for quinine in aqueous solutions of sulfuric acid.

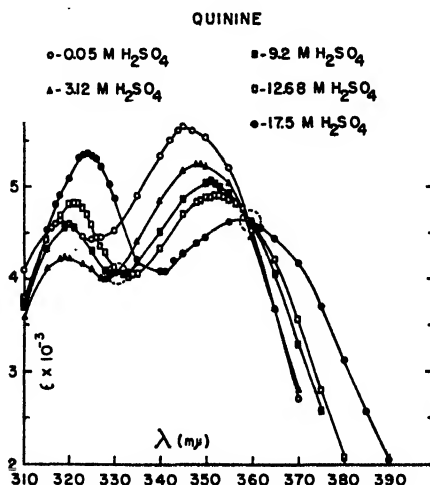


FIG. 3. Spectrophotometric absorption curves for quinine in aqueous solutions of sulfuric acid.

are drawn to a larger scale in Fig. 3 in order to illustrate features of the changes which could not be shown on the smaller scale. The changes are reversible by dilution of the solutions with water. Again, a reversible proton exchange (with the methoxy group as possible acceptor) is sug-

gested, but the absence of true isosbestic points<sup>6</sup> and the unusually wide range of concentrations of sulfuric acid in which the changes in absorption by quinine occur argue in favor of attributing the changes to a "medium effect," at least in part.

Quinine in 0.1 M  $H_2SO_4$  is brilliantly fluorescent. From 0.1 to 3 M sulfuric acid there is a slight increase in the intensity of fluorescence of quinine, possibly due to a medium effect. The intensity of fluorescence of quinine remains the same in sulfuric acid from 3 to 7 M. From 7 to 18 M sulfuric acid there is a great and progressive diminution of fluorescence of quinine, the fluorescence being quite weak in 18 M acid. The mid-point of the change is 12 M sulfuric acid. The change is reversed by dilution of the solutions with water. This change in fluorescence of quinine, occurring

TABLE II  
*Analysis of Mixtures of Quinine and Pamaquine by Measurement of Fluorescence in 0.1 M  $H_2SO_4$  and Concentrated  $H_2SO_4$  (18 M)*

Quantity of pamaquine in mixture	Quantity of quinine in mixture	Concentration of $H_2SO_4$ in final solution	Quantity calculated from fluorescence	
			Pamaquine	Quinine
$\gamma$	$\gamma$	M	$\gamma$	$\gamma$
0	1.3	0.1		1.27
1.26	1.3	0.1		1.31
2.52	1.3	0.1		1.28
5.04	1.3	0.1		1.26
1.26	0	18	1.24	
1.26	1.3	18	1.37	
1.26	2.6	18	1.52	
1.26	5.2	18	1.86	
1.26	13.0	18	2.63	

concomitantly with the change in spectrophotometric absorption, suggests a reversible proton exchange, but a definite conclusion cannot be drawn on the basis of this evidence alone. Practical use can be made of the fact that the changes in fluorescence of quinine and pamaquine in concentrated aqueous solutions of sulfuric acid are in opposite directions. The data of Table II demonstrate that quinine can be determined specifically by measurement of its fluorescence in 0.1 M  $H_2SO_4$ , even in the presence of very large amounts of pamaquine (or SN-13276). On the other hand, pamaquine (or SN-13276) can be determined by measurement of its fluorescence in 18 M sulfuric acid with only minor interference from quinine when the two compounds are present in mixtures in nearly equal proportions. The interference from quinine becomes serious when it is present in much larger

\* The significance of the isosbestic point is discussed by Clark (4).



amounts than pamaquine. However, even in the latter case pamaquine can be estimated from the fluorescence in 18 M  $\text{H}_2\text{SO}_4$  by subtracting the fluorometric value which would be expected from the amount of quinine known to be present in the mixture from specific determination of quinine by measuring fluorescence in 0.1 M  $\text{H}_2\text{SO}_4$ . This is of practical value, inasmuch as quinine and pamaquine (or SN-13276) sometimes are administered together in the treatment of *vivax* malaria.

In the case of SN-3294, a large and progressive increase in fluorescence occurs in solutions of this compound in sulfuric acid from 7 to 18 M. This is the range in which great changes in spectrophotometric absorption by the compound occur (3, 5); the mid-points of the spectrophotometric and fluorometric changes practically coincide. These changes in absorption have been attributed to a reversible proton exchange involving the 4-amino group of SN-3294 as acceptor (3, 5). It appears likely that the change in fluorescence also is due to this proton exchange. In this case evidence for a proton exchange involving the methoxy group is lacking unless the small shift in the wave-lengths of the maxima of the absorption curves observed from approximately 7 to 14 M sulfuric acid is due to such a proton exchange rather than to a "medium effect," as suggested in the preceding paper (3).

#### SUMMARY

A fluorometric method is described for the determination of pamaquine, SN-13276, and SN-3294 in biological samples. The procedure also is applicable to mixtures of any one of these compounds with quinine. The method is based upon the fluorescence of these compounds in concentrated sulfuric acid. Spectrophotometric and fluorometric observations are recorded which suggest alternative explanations for the reversible changes occurring in solutions of these compounds in sulfuric acid.

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# STUDIES OF ARTERIOVENOUS DIFFERENCES IN BLOOD SUGAR\*

## II. EFFECT OF HYPOGLYCEMIA ON THE RATE OF EXTRAHEPATIC GLUCOSE ASSIMILATION

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In a previous paper<sup>1</sup> we described the quantitative relationship between the degree of hyperglycemia and the rate of extrahepatic glucose assimilation in healthy persons. In the present report we present the effect of hypoglycemia upon the rate of extrahepatic glucose assimilation. In order to avoid misunderstanding, it is to be noted that hypoglycemia in our terminology denotes any glycemic level which is below the individual postabsorptive level (fasting blood sugar) in healthy individuals. It will be demonstrated in the course of this report that a drop of but a few mg. per cent below the postabsorptive level actually exerts the physiological effects which we ascribe to hypoglycemia. It must be emphasized, however, that the validity of the results in studies dealing with the A-V<sup>2</sup> differences hinges, to a greater measure than in most experiments involving sugar determinations, upon the accuracy of the analytical work.

The hypoglycemic state during which the observations here reported were made was the well known "spontaneous" hypoglycemia which trails with great regularity in the wake of the alimentary hyperglycemia produced by oral or parenteral administration of glucose. It could scarcely escape attention in our previous experiments<sup>1</sup> that, following the oral administration of glucose, the A-V difference first increased rather rapidly, but then showed a sudden decrease. A rather abrupt dip took place sometimes during the 3rd, more frequently during the 4th, hour after glucose feeding. In a good many cases, however, the A-V difference remained high even at the end of the 4th hour, while in a few instances it receded to its initial (fasting) value as early as the end of the 2nd hour. The change showed no regular relationship to the time element. On closer scrutiny it became apparent that the abrupt decrease in the A-V difference consistently coincided with the time interval during which the arterial blood sugar had fallen below its postabsorptive level. This impression was strengthened by observations on two subjects who remained hyperglycemic and main-

\* This work was aided by the David May-Florence G. May Fund.

<sup>1</sup> Somogyi, M., *J. Biol. Chem.*, **174**, 189 (1948).

<sup>2</sup> A-V = arteriovenous.

tained a broad A-V difference even at the end of the 4th hour. When, however, the test was repeated in these instances and extended to 5 hours, a sudden dip of the A-V difference did develop in the course of the 5th hour, at the same time that the arterial blood sugar declined to a hypoglycemic level.

In further studies of this effect of hypoglycemia upon the rate of extrahepatic glucose assimilation, we fed the subjects 50 gm. of glucose instead of 100 gm. The time of absorption, and hence the duration of the alimentary hyperglycemia, was thus cut shorter, and undue prolongation of individual observations to 4 or 5 hours could be avoided. The results of these experiments, presented in Table I, clearly show the intimate connection between the onset of hypoglycemia and the abrupt decrease of the A-V difference. In four instances (Subjects 1 to 4) hypoglycemia occurred in the course of the 2nd hour after glucose feeding, and so did the dip in the A-V difference. In the next thirteen cases (Subjects 5 to 17) hypoglycemia and the abrupt shrinkage of the A-V difference concurrently took place during the 3rd hour. Finally, in one subject (No. 18) the hyperglycemia extended into the 3rd hour and, along with it, the A-V difference remained high. But then, in the 4th hour, when hypoglycemia occurred, the A-V difference fell to 1 mg. per cent from a height of 25 mg. per cent. There was but a single subject (No. 13) in this series of eighteen in which the sharp dip in the A-V difference lagged somewhat behind the onset of hypoglycemia.

Two details in Table I deserve some comment. One is the fact that the venous blood sugar may decline to a distinctly hypoglycemic level, and yet the A-V difference remains high; this was the case in Subjects 6, 11, 12, 15, and 16, in the 2nd hour after glucose feeding. It is to be noted, however, that during that period hyperglycemia still persisted in the arterial blood. The sharp shrinkage of the A-V difference took place only in the 3rd hour, when the arterial blood sugar, too, had declined to a hypoglycemic level. The second notable detail concerns the extreme delicacy and sensitivity of the mechanism which governs this relationship. This sensitivity is most conspicuous in Subjects 4 and 18, in which arterial hypoglycemias of only 1 and 2 mg. per cent sufficed to initiate the rapid shrinkage of the A-V difference. Conversely, Subjects 11, 12, and 15 show that minimal degrees of arterial hyperglycemia, which exceed the fasting level by only a few mg., can keep the A-V differences lingering at or near the high values to which they had climbed during the preceding hyperglycemic period.

The salient point of our findings, then, is the fact that at various intervals after glucose feeding abrupt shrinkage of the A-V difference takes place, which is intimately linked with the onset of arterial hypoglycemia,

TABLE I

*Showing Sharp Dip in Arteriovenous Difference during Arterial Hypoglycemia  
Following Alimentary Hyperglycemia*

Values in mg. per cent.

Subject No.	Origin of blood	Time intervals after ingestion of glucose (50 gm.)											
		Fasting		0.5 hr.		1 hr.		2 hrs.		3 hrs.		4 hrs.	
		Blood sugar	A-V	Blood sugar	A-V	Blood sugar	A-V	Blood sugar	A-V	Blood sugar	A-V	Blood sugar	A-V
1	Arterial*	86		184		127		72		83		88	
	Venous	84	2	164	20	90	37	66	6	81	2	87	1
2	Arterial	79		153		135		52		69		77	
	Venous	78	1	113	40	103	32	49	3	67	2	76	1
3	Arterial	89		154		146		83		62		80	
	Venous	82	7	138	16	126	20	78	5	61	1	78	2
4	Arterial	95		185		152		93		85		86	
	Venous	88	7	157	28	134	18	88	5	81	4	84	2
5	Arterial	90		132		111		107		70		77	
	Venous	81	9	98	34	83	28	84	23	66	4	76	1
6	Arterial	92		193		129		122		81		88	
	Venous	88	4	157	36	95	34	84	38	75	6	85	3
7	Arterial	89		153		181		112		66		75	
	Venous	86	3	136	17	142	39	91	21	65	1	74	1
8	Arterial	93		142		177		121		71		77	
	Venous	91	2	134	8	142	35	101	20	69	2	77	<1
9	Arterial	84		131		156		120		62		69	
	Venous	79	5	114	17	128	28	100	20	59	3	66	3
10	Arterial	102		170		167		127		67		80	
	Venous	96	6	153	17	146	21	108	19	65	2	79	1
11	Arterial	91		180		180		91		64		77	
	Venous	85	6	143	37	151	29	75	16	62	2	72	5
12	Arterial	94		152		152		97		83		88	
	Venous	90	4	131	21	124	28	83	14	82	1	83	5
13	Arterial	92		168		114		81		88		87	
	Venous	85	7	142	26	86	28	67	14	84	4	85	2
14	Arterial	88		167		154		123		70		82	
	Venous	87	1	153	14	137	17	110	13	69	1	78	4
15	Arterial	94		149		145		100		81		85	
	Venous	90	4	128	21	123	22	87	13	80	1	81	4
16	Arterial	91		168		171		92		70		83	
	Venous	89	2	153	15	154	17	79	13	67	3	79	4
17	Arterial	87		136		169		126		67			
	Venous	81	6	123	13	158	11	112	14	66	1		
18	Arterial	79		88		122		116		112		78	
	Venous	74	5	83	5	105	17	89	27	87	25	77	1

\* Capillary finger blood was used to obtain the values listed opposite arterial. This procedure has been shown (see foot-note 1) to provide a satisfactory substitute for arterial puncture.

but is independent of the time that has elapsed after the ingestion of glucose. This phenomenon appears significant enough to merit additional\* illustration and demonstration. For this purpose we have divided the cases presented in Table I into two groups, according to the time of the onset of the hypoglycemic state. In Table II are presented the average values of their glycemic levels and A-V differences (Groups 1 and 2). Group 3 in Table II represents the average values of experiments in which ten individuals were fed 100 gm. of glucose each. These were picked as examples from a previous study.<sup>1</sup> In Group 4 of Table II are given the

TABLE II

*Showing That Sharp Dip in A-V Difference Is Chronologically Contingent Only on Onset of Hypoglycemia but Unrelated to Time of Glucose Feeding*

The glucose values represent mg. per 100 cc. of blood.

Group No.	Subjects used for averages	Origin of sugar values	Average blood sugar values at intervals after ingestion of glucose						
			Fasting	0.5 hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.
1	Subjects 1 to 4, 50 gm. glucose by mouth	Arterial blood	87	169	160	75	75	83	
		A-V difference	4.3	26	27	4.7	2.3	1.5	
2	Subjects 5 to 16, 50 gm. glucose by mouth	Arterial blood	92	159	153	108	73	81	
		A-V difference	4.4	22	27	19	2.5	2.8	
3	10 subjects, 100 gm. glucose by mouth	Arterial blood	88	151	143	119	115	74	
		A-V difference	2.8	26	28	25	18	3.9	
4	2 subjects, 100 gm. glucose by mouth	Arterial blood	91	157	170	143	147	121	71
		A-V difference	4.0	35	35	30	30	30	2.5
5	1 subject, 100 gm. glucose intravenously*	Arterial blood	92	175	169	240	61	81	
		A-V difference	3.3	10.5	19.7	59.4	1.4	0.3	

\* The infusion lasted 2 hours; in the 1st hour 28 gm., in the 2nd hour 72 gm. of glucose were infused.

average values of two cases in which hyperglycemia persisted as long as 4 hours after the ingestion of 100 gm. of glucose. Finally, Group 5 concerns a healthy young man who received 100 gm. of glucose intravenously in the course of 2 hours, 28 gm. in the 1st and 72 gm. during the 2nd hour. Attention is called to the figures which are boxed in squares. The first square contains the fasting arterial glycemic level; the second encloses the last hour during which hyperglycemia still persisted; finally, the third square presents the interval immediately following the onset of the hypoglycemic state. With each blood sugar level the corresponding A-V difference is given. This arrangement of the data clearly exposes the close, apparently causal, connection between the onset of hypoglycemia in arterial blood and a rapid contraction of the A-V difference.

This rule is most conspicuously demonstrated in Group 5 of Table II. It represents an experiment in which, by means of intravenous administration of glucose, hyperglycemia in a healthy young man was raised to an abnormal height, and then, beginning at the moment when the glucose infusion was terminated, a steep decline of the glycemic level ensued. Owing to limitations imposed by the processes of intestinal absorption, no such sharp and extensive swings of the glycemic level can be produced by oral feeding of glucose. By intravenous administration, on the other hand, it was possible to push the arterial hyperglycemia up to 240 mg. per cent, and then, by terminating the influx of glucose at that very moment, permit the glycemic level to drop sharply to 61 mg. per cent within an hour. The high glycemic level entailed an increase of the A-V difference to 59 mg. per cent. But when the blood sugar dipped below its fasting level in the course of the next hour, the A-V difference precipitously shrank to 1.4 mg. per cent, less than half its fasting value.

To round out the evidence demonstrating the causal connection between the occurrence of hypoglycemia and the decrease of A-V difference, the results of one more experiment are presented here graphically. It is shown in this experiment that when the onset of a hypoglycemia can be anticipated and successfully forestalled the shrinkage of the A-V difference also is prevented. In the first stage of the experiment the subject, a healthy young man, was fed 75 gm. of glucose by mouth and the arterial and venous glycemic levels were observed for 3 hours; in this manner it was established that the usual contraction of the A-V difference took place in the course of the 2nd hour. Next the subject was fed 75 gm. of glucose as previously, but an hour and a half later he was given a second 75 gm. portion of glucose for the prevention of hypoglycemia. As is shown in Fig. 1, the results are fully in line with the rule. The second dose of glucose intercepted the decline of the glycemic level at the proper time to prevent hypoglycemia. Instead, the second dose of glucose effected a slight rise in the arterial glucose level. As a consequence, the A-V difference substantially expanded during the 2nd and 3rd hours (33 mg. per cent), whereas in the first test in this experiment, in which hypoglycemia was permitted to develop, a shrinkage to 3 mg. per cent had taken place during the same period (see Fig. 1).

The curves in Fig. 1 again give warning against two possible errors. First, they show how misleading it would be to correlate the changes in the A-V difference with the time element in the process (as was actually done by a few workers). Secondly, the glucose curves clearly convey the lesson that it is the changes of the arterial, and not of the venous, blood sugar level which must be taken into consideration in evaluating the relationship between glycemic levels and A-V differences. Inspection

of the arterial and venous curves of the second test (Fig. 1) makes this quite obvious: the greatest A-V difference made its appearance after the 3rd hour of the experimental period, when the arterial blood still lingered at a hyperglycemic level; the fact that the venous blood sugar already had dipped to a hypoglycemic level evidently had no effect upon the A-V difference.

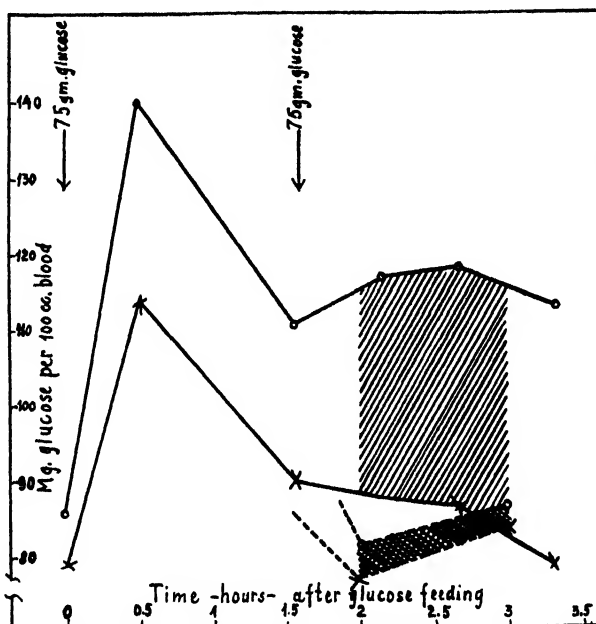


FIG. 1. Illustrating that the shrinkage of A-V difference can be forestalled by preventing the onset of hypoglycemia. Dashed line, blood sugar after a single dose of 75 gm. of glucose (first test); solid line, blood sugar after two 75 gm. doses of glucose (second test); O arterial, X venous. The shaded area shows that the A-V difference remains great so long as arterial hyperglycemia is maintained; the cross-hatched area shows the conspicuous shrinkage of the A-V difference in the same subject as soon as the arterial blood sugar falls below its fasting level.

The effect of arterial hypoglycemia is in diametric contrast to the effect of hyperglycemia, which, as is known, enhances the rate of extrahepatic (as well as hepatic) glucose assimilation. In keeping with accepted concepts and experimental proof, the response to hyperglycemia can be attributed to an increased insulin supply that is brought about by the stimulating effect of hyperglycemia upon the islands of Langerhans. It is not so simple to explain why hypoglycemia entails an abrupt depression in the rate of assimilation. Decrease of the insulin supply would readily be made accountable for it by the (still numerous) proponents of the over-

simplified "unitarian" school of thought. This, however, is untenable in view of the fact that the shrinkage of the A-V difference occurs just at the time when the onset of hypoglycemic states indicates the presence of a greater than normal insulin supply.

A more apt interpretation can be based on the fact, often demonstrated in animal experiments, that insulin supply and insulin action do not always run parallel. Two categories of factors are known which depress insulin action: dietary and endocrine factors. High fat-low carbohydrate diets and acid ash diets are well recognized members of the first group. In the second category belong the several insulin-antagonistic constituents of the pituitary-adrenal system, and probably some other endocrine products; and still there may exist some as yet unrecognized insulin antagonists. In looking for inhibitors of insulin action which are ushered in by hypoglycemia, dietary factors are obviously to be disregarded and one must direct attention to the likelihood that hypoglycemia stimulates to increased activity the endocrine organs which produce the insulin-inhibiting hormones. Prompt response of these organs to hypoglycemia, by supplying increased quantities of insulin inhibitors, would readily account for the abrupt drop in the rate of glucose assimilation. This subject will be treated in greater detail in a forthcoming paper dealing with the effects of insulin on the rate of extrahepatic glucose assimilation.

In conclusion, we wish to call attention to the substantial contribution of the extrahepatic tissues to the homeostasis of the glycemic level during hypoglycemic states. This function is performed by virtue of the fact that the rate of glucose assimilation sharply drops at the onset of hypoglycemia and thus retards the depletion of blood sugar.

#### SUMMARY

Differences between glucose concentrations in arterial (capillary) and venous blood of healthy persons were studied during hypoglycemic states which follow the hyperglycemia produced by glucose feeding.

It was found that the onset of arterial hypoglycemia entails an abrupt drop in the rate of extrahepatic glucose assimilation. This fact is reflected in a sudden shrinkage of the A-V differences, which frequently dip below their postabsorptive (fasting) values; they may decline to less than 1 mg. per cent, but never become negative.

The venous blood sugar may fall to hypoglycemic levels without affecting the A-V difference; it requires the impact of arterial hypoglycemia to depress the rate of glucose assimilation.

The extrahepatic tissues contribute substantially to the homeostasis of the glycemic level by virtue of the decline in their rate of glucose assimilation during hypoglycemia.





# FURTHER STUDIES ON THE RÔLE OF VITAMIN B<sub>6</sub> IN THE METABOLISM OF TRYPTOPHAN BY THE RAT\*

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Recent studies have demonstrated that vitamin B<sub>6</sub>-deficient rats and mice, in contrast to animals receiving an adequate quantity of the vitamin, excrete small amounts of nicotinic acid and N<sup>1</sup>-methylnicotinamide when tryptophan is fed (1, 2). This has been interpreted to mean that vitamin B<sub>6</sub> is necessary for the metabolic conversion of dietary tryptophan to nicotinic acid and its methylated derivative.

It was of interest to extend these observations and to determine the rate at which vitamin B<sub>6</sub>-deficient rats recovered the ability to convert tryptophan to nicotinic acid when the vitamin was again administered. Further, the comparative effectiveness of the three vitamin B<sub>6</sub> derivatives, pyridoxine, pyridoxal, and pyridoxamine, was also studied with animals previously depleted of vitamin B<sub>6</sub> by the use of two criteria, the amounts of nicotinic acid derivatives excreted when tryptophan was fed and the rates of gain.

## EXPERIMENTAL

Weanling male albino rats 40 to 50 gm. in weight (Sprague-Dawley strain) were used in these studies. A basal ration was fed consisting of sucrose 67, purified casein 24, Salts IV (3) 4, corn oil 4.7, and vitamins A and D concentrate 0.3 per cent, plus the following amounts of B vitamins per 100 gm. of ration: thiamine 250  $\gamma$ , riboflavin 300  $\gamma$ , calcium pantothenate 2 mg., inositol 100 mg., choline 100 mg., pteroylglutamic acid 200  $\gamma$ , and biotin 10  $\gamma$ . Control groups received 250  $\gamma$  of pyridoxine hydrochloride per 100 gm. of ration in addition to the basal ration. After 3 weeks on experiment, quantitative urine collections were made, and the amounts of nicotinic acid and N<sup>1</sup>-methylnicotinamide excreted per day were determined by techniques described in earlier work (1).

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Pooled urine collections for 2 or 3 day periods were obtained when the basal ration was fed and also when the basal ration plus 100 mg. of DL-tryptophan per day was fed for the subsequent 2 to 3 day period. This sequence of urine collections was repeated until the inability of the vitamin B<sub>6</sub>-deficient rats to convert tryptophan to nicotinic acid and N<sup>1</sup>-methyl-nicotinamide as determined by the urinary excretion was demonstrated.

In the first experiments, the effect of supplementing the diet of vitamin B<sub>6</sub>-deficient rats on the conversion of tryptophan to nicotinic acid and N<sup>1</sup>-methyl-nicotinamide was determined after the rats had been fed the

TABLE I

*Effect of Recovery from Vitamin B<sub>6</sub> Deficiency on Urinary Excretion of Nicotinic Acid and N<sup>1</sup>-Methylnicotinamide by Rats Fed Tryptophan*

The values are expressed as micrograms excreted per rat per day.

Dietary regimen	Experiment I			Experiment II		
	Days on experiment	Nicotinic acid	N <sup>1</sup> -Methyl-nicotinamide	Days on experiment	Nicotinic acid	N <sup>1</sup> -Methyl-nicotinamide
Basal ration (-B <sub>6</sub> )	41-43	7.8	35	35-37	12.5	53
" " + tryptophan*	53-56	10.8	34	38-40	25.0	235
" " + B <sub>6</sub> †	57-58	14.1	50	41-43	9.4	57
" " + " + tryptophan	59-60	32.1	256	44-46	50.0	1327
" " + "	61-63	22.7	60	47-49	9.4	118
" " + " + "	64-66	59.9	645	50-52	95.8	3240
" " + " + "	67-68	21.7	144	53-55	11.4	640
" " + " + "				56-58	83.3	1651
" " + " + "				59-60	20.3	381
" " + " + "				61-62	81.3	1930

\* In all cases 100 mg. of DL-tryptophan were fed per rat per day.

† 250 γ of pyridoxine hydrochloride per 100 gm. of ration.

vitamin B<sub>6</sub>-deficient diet for a period of 56 days (Table I). In these studies vitamin B<sub>6</sub> was supplied as 250 γ of pyridoxine hydrochloride per 100 gm. of ration. In a subsequent experiment animals that had been fed the deficient diet for 40 days were used. Each figure represents the average of the values obtained for four rats used in each sequence of dietary treatment.

For the studies to determine the comparative effectiveness of pyridoxine, pyridoxal, and pyridoxamine, the animals were fed the deficient diet for 40 days. Since variations in food consumption would tend to increase the variability in the recovery of the animals when the test compounds were added to the ration, these tests were carried out by injecting the vitamin B<sub>6</sub> derivatives intraperitoneally. 25 γ of pyridoxine hydro-

chloride or the equivalent of the other derivatives on a molar basis were injected daily. This level was selected since it approximates the minimum daily requirement of the rat for vitamin B<sub>6</sub> and would give a more critical comparison of the three vitamin B<sub>6</sub> derivatives than if greater amounts were given. Four vitamin B<sub>6</sub>-deficient rats were used in each group, and the results obtained for the three groups before and after vitamin supplementation and the sequence of tryptophan feeding are shown in Table II.

TABLE II

*Comparative Effect of Injecting Pyridoxine, Pyridoxal, or Pyridoxamine on Urinary Excretion of Nicotinic Acid and N<sup>1</sup>-Methylnicotinamide by Rats Fed Tryptophan*

All the values are expressed as micrograms excreted per rat per day.

Dietary regimen	Days on experiment	Nicotinic acid	N <sup>1</sup> -Methylnicotinamide	Nicotinic acid	N <sup>1</sup> -Methylnicotinamide	Nicotinic acid	N <sup>1</sup> -Methylnicotinamide
basal ration (-B <sub>6</sub> )	35-37	15.0	108	11.0	68	10.8	54
" " + tryptophan*	38-40	10.6	298	21.3	210	17.7	314
		25 γ or equivalent injected per day†					
		Pyridoxine		Pyridoxal		Pyridoxamine	
basal ration	41-43	12.4	96	9.8	94	10.4	99
" " + tryptophan	44-46	36.5	1002	34.4	1191	45.2	1255
" " + "	47-49	21.3	258	16.4	290	15.9	243
" " + "	50-52	115.6	2814	79.1	2749	97.9	2502
" " + "	53-55	26.2	423	19.6	450	9.8	349
" " + "	56-58	149.5	2540	158.2	1440	103.3	1246
" " + "	59-60	22.5	430	22.0	392	17.2	380
" " + "	61-62	68.8	2125	90.6	2135	61.9	1540

\* 100 mg. of DL-tryptophan per rat per day.

† Calculated as pyridoxine hydrochloride on a molar basis. Pyridoxine and pyridoxal were given as the hydrochloride and pyridoxamine as the dihydrochloride.

## RESULTS AND DISCUSSION

In the present work the inability of vitamin B<sub>6</sub>-deficient rats to excrete relatively large amounts of nicotinic acid and N<sup>1</sup>-methylnicotinamide when tryptophan was fed has been confirmed. For example, after 26 days on experiment, rats deficient in vitamin B<sub>6</sub> were fed 100 mg. of DL-tryptophan per day and excreted 13.3 γ of nicotinic acid daily, whereas the vitamin B<sub>6</sub>-supplemented group excreted 374.8 γ per rat daily. The corresponding values for N<sup>1</sup>-methylnicotinamide were 275 and 3817 γ respectively. Similar data obtained in other experiments are shown in Tables I and II.

The rate of recovery from the vitamin B<sub>6</sub> deficiency when pyridoxine was added to the ration, as measured by the ability of the rat to convert tryptophan to nicotinic acid and N<sup>1</sup>-methylnicotinamide, is given in Table I. It was clearly shown that the stage of the deficiency was an important factor that influenced the rate of recovery. For example, when vitamin B<sub>6</sub> supplementation began after 56 days on the deficient diet, 7 days later the excretion of nicotinic acid and N<sup>1</sup>-methylnicotinamide was only 60 and 645  $\gamma$  respectively, when rats were fed 100 mg. of DL-tryptophan per day. However, when pyridoxine supplementation was initiated on the 41st day of the deficiency, the respective values after 9 days of vitamin B<sub>6</sub> supplementation were 95.8 and 3240  $\gamma$ . Subsequent experiments conducted by injecting pyridoxine or related compounds also demonstrated that the recovery of the animals in terms of the excretion of nicotinic acid and N<sup>1</sup>-methylnicotinamide was much more rapid when the animals were fed the deficient diet for only 40 days, as compared to the results presented in Table I for animals fed the deficient diet for 56 days. These results were consistent for all tests conducted with each group in each experiment.

Rosen and associates (2) stated that even after 2 weeks of daily administration of 50  $\gamma$  of vitamin B<sub>6</sub> to rats which had been fed a vitamin B<sub>6</sub>-deficient diet for 31 days they were unable to detect an appreciable increase in the amounts of N<sup>1</sup>-methylnicotinamide excreted when tryptophan was fed. These workers used a higher level of casein (35 per cent as compared to 24 per cent) and the ingestion of higher levels of casein accentuates a deficiency of vitamin B<sub>6</sub> (4-6); therefore their animals may have been more deficient in vitamin B<sub>6</sub> than the animals used in the present studies.<sup>1</sup> Whether these variations in the effects noted can be attributed to the activity of the intestinal flora or to the ability of the tissues to carry out the transformations involved or to a combination of these factors cannot be determined at present.

It can be seen from the data presented in Table II that pyridoxine, pyridoxal, and pyridoxamine are essentially equal in their effectiveness and the recovery from the deficiency was very rapid. There was a large increase in the amounts of nicotinic acid and N<sup>1</sup>-methylnicotinamide excreted when tryptophan was fed during the 4th to 6th days of vitamin supplementation. After the 10th day of supplementation the values for the amounts of nicotinic acid and N<sup>1</sup>-methylnicotinamide excreted can be considered normal when tryptophan was fed (compare also with the data in Table I and in previous papers (1, 8)). Other animals fed the vitamin

<sup>1</sup> Since this paper was prepared for publication, Bell *et al.* (7) have reported that rats which had received a vitamin B<sub>6</sub>-deficient diet (15 per cent casein) for 22 days excreted large amounts of N<sup>1</sup>-methylnicotinamide in particular, when tryptophan was fed 8 days after pyridoxine supplementation was initiated.

B<sub>6</sub>-deficient diet continued to excrete small amounts of nicotinic acid and N<sup>1</sup>-methylnicotinamide when tryptophan was fed.

The conclusion that pyridoxine, pyridoxal, and pyridoxamine are equally effective for the rat when injected is in agreement with the work of Snell and associates (9, 10). The criterion for comparison used by the latter workers was the rate of growth. Growth data were also secured in the present experiments and, during the 3 weeks of recovery from the deficiency, the gains obtained were as follows for the pyridoxine-, pyridoxal-, and pyridoxamine-supplemented groups: 21.0, 22.3, and 20.7 gm. per week respectively, whereas the control group which received no supplement grew at a rate of 1.5 gm. per week. It appears therefore that the rate of transformation of pyridoxine and pyridoxamine to pyridoxal, recognized as the biologically active form of the vitamin, is sufficiently rapid to permit equal effectiveness for all three compounds to be demonstrated when measured either by the comparative rates of gain or excretion of nicotinic acid derivatives.

The mechanism by which vitamin B<sub>6</sub> functions in the conversion of tryptophan to nicotinic acid has not as yet been established. Indole, indoleacetic acid, indolepropionic acid, indolebutyric acid, and at least certain amino acids other than tryptophan are inactive precursors of nicotinic acid for the rat, judged on the basis of the excretion of nicotinic acid and N<sup>1</sup>-methylnicotinamide when the test compounds were fed to animals receiving adequate diets (8, 11, 12).<sup>2</sup> Rosen and associates (2) have reported that kynurenine, kynurenic acid, and xanthurenic acid, all metabolites of tryptophan, are inactive as precursors of N<sup>1</sup>-methylnicotinamide when tested with rats receiving adequate diets. A recent report by Beadle *et al.* (13) demonstrates that kynurenine is an intermediate in the formation of nicotinic acid from tryptophan by *Neurospora*. This suggests that further studies with the rat and other animals may reveal some information on the pathway whereby tryptophan is converted to nicotinic acid and which metabolic reaction vitamin B<sub>6</sub> influences. From the known alterations in the metabolism of tryptophan by the B<sub>6</sub>-deficient rat and the work of Beadle *et al.* (13), it would appear that an exhaustive study of the effects of kynurenine, kynurenic acid, and xanthurenic acid on nicotinic acid formation would be very valuable.

The possibility that the inability of rats to convert tryptophan to nicotinic acid derivatives also occurs with animals deficient in dietary factors other than vitamin B<sub>6</sub> cannot be excluded. Preliminary experiments have indicated that at least some reduction in the excretion of nicotinic acid metabolites occurred when tryptophan was fed to riboflavin- or thiamine-deficient rats, as compared to the results obtained with rats fed ade-

<sup>2</sup> Schweigert, B. S., unpublished data.

quate diets. These studies are being extended to obtain more information on the specificity of a vitamin B<sub>6</sub> deficiency on the inability of rats to transform tryptophan to nicotinic acid and its methylated derivative.

#### SUMMARY

Studies have been made on the rate of recovery of vitamin B<sub>6</sub>-deficient rats after supplementation with pyridoxine, as measured by the amounts of nicotinic acid and N<sup>1</sup>-methylnicotinamide excreted in the urine when 100 mg. of DL-tryptophan were fed per day. A comparison of the relative activities of pyridoxine, pyridoxal, and pyridoxamine measured by the rate of gain and also by the amounts of nicotinic acid and N<sup>1</sup>-methylnicotinamide excreted in urine when tryptophan was fed was also conducted.

The rate of recovery of vitamin B<sub>6</sub> deficiency, when pyridoxine hydrochloride was fed mixed in the ration was dependent on the degree of deficiency of the rat. The addition of 250  $\gamma$  of pyridoxine hydrochloride per 100 gm. of ration on the 41st day of deficiency for a period of 10 days restored the ability of the rats to excrete normal amounts of nicotinic acid and N<sup>1</sup>-methylnicotinamide when tryptophan was ingested. The increase noted in the excretion of the nicotinic acid compounds was much smaller, however, when pyridoxine supplements were given to rats maintained for 56 days on the vitamin B<sub>6</sub>-deficient diet as compared to the results obtained for animals maintained 41 days on the deficient diet.

Pyridoxine, pyridoxal, and pyridoxamine were found to be equally effective in restoring the ability of vitamin B<sub>6</sub>-deficient rats to convert tryptophan to nicotinic acid and in promoting gains in weight.

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# A CHICK GROWTH FACTOR IN COW MANURE

## VII. ITS STABILITY AND SOLUBILITY

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A previous report (1) described methods of extracting and concentrating a new unidentified growth factor from cow manure, and summarized the available information on the properties of the factor. Later it was reported (2) that the same concentrates which counteracted the deficiency in the chick starting diet also counteracted the deficiency in a similar diet for breeding birds and permitted satisfactory hatchability. These concentrates obviously contained much inert material, and subsequent fractionation experiments to be described in this paper indicated that some of the apparent properties of the factor were due to its association with protein and were not characteristic of the active factor itself.

### EXPERIMENTAL

The fraction which precipitated at pH 3.0 from a water extract of dried cow manure was prepared as previously described (1). The procedures to which this material was subjected are indicated in Table I.

*Precipitation with  $(\text{NH}_4)_2\text{SO}_4$*  was accomplished as previously described (1), except that in some cases the concentration of  $(\text{NH}_4)_2\text{SO}_4$  was varied, as noted in Table I.

*Precipitation with 2 Per Cent  $\text{CaCl}_2$* —5 gm. of acid precipitate were suspended in distilled water and dissolved by adjusting the pH to 6.8. The volume was then adjusted to 250 ml. 5 gm. of  $\text{CaCl}_2$  were dissolved in 25 ml. of water and added to the above-mentioned solution. The pH was readjusted to a value between 6.5 and 6.9. The precipitate was removed either by centrifugation or by filtration and washed twice by suspending in 2 per cent  $\text{CaCl}_2$  solution. The solution remaining after removal of the precipitate was, in several cases, fractionated further. In one case a dialysate was prepared from it with a cellophane membrane. In another case, 1 volume of the solution was mixed with 5 volumes of 95 per cent ethanol. A brown gelatinous precipitate formed which was separated from the solution by filtration. In a third case the  $\text{CaCl}_2$ -soluble fraction was treated with acetone, as described below. In several cases the  $\text{CaCl}_2$ -soluble fraction was freed of Ca by adding a concentrated solution of  $\text{Na}_2\text{CO}_3$  to supply approximately 2 gm. of  $\text{Na}_2\text{CO}_3$  per 100 ml. of solution. The  $\text{CaCO}_3$  was removed by filtration.



TABLE I  
*Stability and Solubility of Growth Factor Found in Cow Manure*

	Potency index* of preparation fed equivalent to level of acid ppt. in positive control diet unless otherwise noted							
	Preparation No.							
	1	2	3	4	5	6	7	8
Effect of autoclaving and pH on stability								
Acid ppt. autoclaved 2 hrs., pH 7.0	192							
pH 3.0 solubles of above	-43							
" 3.0 ppt. of above	100							
Acid ppt. autoclaved 2 hrs., pH 8.5		0						
pH 3.0 solubles of above		15						
" 3.0 ppt. of above		81						
Acid ppt. autoclaved 1 hr., 2 N HCl			-111†					
Effect of (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> on solubility								
Half saturated (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> solubles	164	80‡		24			34	
pH 2.0 ppt. of above			23		48§	-143		
" 2.0 solubles of above (non-dialyzable)			93			-231		
Half saturated (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ppt.	133	4‡	-30				104	
pH 3.0 ppt. of above						135		
43% saturated (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> solubles								13
43% saturated (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ppt.								42
33% saturated (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> solubles								22
33% saturated (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ppt.								51
Effect of 2 per cent CaCl <sub>2</sub> on solubility								
CaCl <sub>2</sub> solubles	85		69, 33					
Dialysate of above		29						
95% EtOH solubles of above			29					
95% EtOH ppt. of above			13					
80% acetone solubles of above			-3, +12					
80% acetone ppt. of above			33, 20					
Ca-free solution of above			31	167	-29§			

TABLE I—Continued.

	Potency index* of preparation fed equivalent to level of acid ppt. in positive control diet unless otherwise noted							
	Preparation No.							
	1	2	3	4	5	6	7	8
Effect of 2 per cent $\text{CaCl}_2$ on solubility—continued								
$\text{CaCO}_3$ ppt. of above			16†		59¶			
pH 3.0 solubles of above (Ca-free)			44					
pH 3.0 ppt. of above (Ca-free)			1					
$\text{CaCl}_2$ ppt.	50							
Dialysate of above		7						
Solubility in 80 per cent acetone								
80% acetone solubles (1st and 2nd fractions)	119	78	101					
80% acetone solubles (1st fractionation)			107, 95					
pH 3.0 solubles of above			31					
" 3.0 ppt. of above			82					
80% acetone ppt. (1st fractionation)								
80% acetone solubles of above (2nd fractionation)			66, 58					
pH 3.0 solubles of above			81					
pH 3.0 ppt. of above			62					
80% acetone ppt. of above (2nd fractionation)	50	0	35					
Effect of papain digestion on solubility								
Papain digest	92	138						
pH 3.0 ppt. of above	—2†							
" 3.0 solubles of above	77	123†	53§					
Norit filtrate of above		23†						
MeOH solubles of above		87**						
MeOH ppt. of above		43**						
Norit eluate of above		28†						
Ppt. upon heating above to 100°			22§	52§				
Solution upon heating above to 100°			121§	109§				

TABLE I—*Concluded*

Potency index\* of preparation fed equivalent to level of acid ppt. in positive control diet unless otherwise noted

Preparation No.

Solubility in ethanol at different pH values

Ammoniacal EtOH-insoluble fraction	-9	4††						
Ammoniacal EtOH solubles	77	83††						
Neutral EtOH-insoluble fraction			92					
Ammoniacal EtOH-insoluble fraction of above				-36††	2††	66††		
Ammoniacal EtOH-soluble fraction of above				-39††		24††		
pH 3.0 ppt. of above				44††				
" 3.0 solubles of above				52††				
Neutral EtOH solubles				61††	39††	32††		
Fraction ppt. at 2°			7					
" soluble at 2°			43					
Acid (pH 4.0) EtOH-insoluble fraction							61††	133††
Acid (pH 4.0) EtOH solubles							3††	-20††

(Weight supplemented group - weight negative control)  
(Weight positive control - weight negative control)  $\times 100$ .

† Equivalent to level of acid precipitate  $\times 1.5$ .

‡ Equivalent to level of acid precipitate  $\times 0.33$ .

§ Equivalent to level of acid precipitate  $\times 2$ .

|| Equivalent to level of acid precipitate  $\times 1.2$ .

¶ Fed as 1 per cent of the diet.

\*\* Equivalent to level of acid precipitate  $\times 4$ .

†† Obtained by 1 week assay; all other figures obtained by 4 week assay.

*Precipitation from 80 Per Cent Acetone Solution*—30 gm. of acid precipitate were dissolved in 1500 ml. of water at pH 7.0. 6 liters of acetone were added, 2 liters at a time, with shaking after each addition. The mixture was allowed to stand overnight and the precipitate was then removed by filtering with suction. The acetone solution was concentrated in a vacuum still at 40°. It contained approximately 20 gm. of solid matter. The precipitate was dissolved in 1 liter of water and reprecipitated with 4 liters of acetone, added in three portions, with shaking. The properties of this second precipitate were different from those of the first. The second

precipitate was darker in color, settled more slowly, and could not be separated by filtration. A portion of the supernatant liquid was removed by siphoning and the remainder was separated from the precipitate by centrifugation. The second acetone solution was concentrated in the same manner as the first. The second precipitate, after drying in a vacuum oven at 45°, weighed 5 gm.

*Digestion with Papain*—The acid precipitate was dissolved in water at pH 7.0, the concentration being 2 gm. of acid precipitate per 100 ml. of solution. The solution was adjusted to pH 5.0 with HCl. The slight precipitate which formed was separated by centrifugation. The solution was divided into 250 ml. portions, each of which was placed in a 500 ml. Erlenmeyer flask with 25 gm. of papain. Toluene was added and the flasks were incubated at 37° for 48 hours with periodic shaking. The contents of the flasks were combined, and the undissolved enzyme preparation was separated by centrifugation and twice washed by resuspending in water and centrifuging. The original supernatant was filtered with suction to remove a small amount of very fine suspended matter and was then combined with the washings. A portion of the combined solution was acidified to pH 3.0, and the resulting precipitate was removed by centrifugation. The remaining solution was divided into two equal parts, one of which was stirred for 90 minutes with 10 gm. of norit. The norit was allowed to settle for 5 minutes and then removed by suction filtration. The norit was washed once with water and the washings combined with the original filtrate. The norit and filter paper were covered with 700 ml. of 10 per cent  $\text{NH}_4\text{OH}$  and stirred for 5 hours at room temperature. The norit was separated from the eluate on a suction filter and washed with 10 per cent  $\text{NH}_4\text{OH}$ . The combined eluate and washings were boiled for 3 hours to remove  $\text{NH}_3$ .

One portion of eluate was concentrated to a sirup and treated with a large excess of absolute methanol. A white precipitate formed and was separated by filtration with suction.

*Extraction with Ethanol*—To 9 gm. of acid precipitate in a round bottom flask was added 1 liter of 95 per cent ethanol. The mixture was heated 2 to 3 hours at 65–70° under a reflux condenser, and then filtered. The residue was washed twice with 50 ml. portions of hot ethanol and then returned to the flask and refluxed with a second, and then with a third, 1 liter portion of ethanol. The final residue, after drying, weighed 4.7 gm. The combined extracts were allowed to stand 3 days at 2°. A precipitate formed which was removed by centrifugation. The supernatant liquid was concentrated under reduced pressure.

Extraction with ammoniacal ethanol was accomplished in much the same way as outlined above, except that the solvent consisted of 900 ml.

of 95 per cent ethanol and 100 ml. of concentrated  $\text{NH}_4\text{OH}$ . Two extractions were made, each of 4 hours duration. The residue weighed 5.4 gm., or 60 per cent of the original weight of the acid precipitate.

The combined extracts were concentrated (in one case at  $90^\circ$  and in another at  $70^\circ$ ) under reduced pressure to a sirup and then to dryness on a steam bath.

*Testing of Preparations*—Most of the testing was done by the method described by Rubin and Bird (1). However, in the later stages of the work, a short test was suggested by the finding that high levels of soy bean meal increased the requirement for the factor (3) and by the report of Zucker *et al.* (4) that weight differences were observed within 2 or 3 days after effective supplements were given to young rats fed a diet high in vegetable protein. In the short test, as in the older method, chicks were obtained from hens fed a diet low in the essential factor, with the additional precaution that all hens producing eggs of high hatchability when fed this diet were eliminated. It has been shown that the progeny of such hens are not very susceptible to the deficiency (5). All chicks were fed the basal diet until 2 weeks old and then groups of twelve birds, carefully equalized with respect to weight and ranging in individual weight from 65 to 90 gm., were placed in cages in an air-conditioned room at  $25.5^\circ$  and continued on the basal diet, except that each test group received as a supplement one of the fractions to be tested. In each experiment a negative control group was fed the basal diet alone and a positive control group the basal diet plus 0.15 per cent of the acid precipitate fraction. The composition of the basal diet was yellow corn 23.0 per cent, alfalfa leaf meal 3.0, soy bean meal 70.0, butyl fermentation solubles (containing 250  $\gamma$  of riboflavin per gm.) 0.6, steamed bone meal 1.5, limestone 1.0, salt (96 per cent  $\text{NaCl}$ , 4 per cent  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ ) 0.7, and vitamins A and D feeding oil (400 A. O. A. C. units of vitamin D, 2000 U. S. P. units of vitamin A per gm.) 0.2. 1 mg. of nicotinic acid was added to each 100 gm. of diet.

When the short test was first tried good results were obtained in 1 week in a number of cases. The results of several of these tests are included in Table I. Recently the response of chicks to effective supplements has been slower, making it necessary to resort to a test period of 2 weeks. Even this is a considerable improvement over the older method, which required 4 weeks.

#### RESULTS AND DISCUSSION

The effects of the various fractions upon growth of chickens are summarized in Table I. To facilitate comparison of the results of different tests, all results were expressed as "potency indices" rather than as average weights. The method of calculating the potency index is indicated below

Table I. An index of 0 indicates that the growth of chicks fed the supplemented diet was no better than the growth of chicks fed the basal diet. Supplements having an index of 100 were as effective as the original acid precipitate in equivalent quantities. Supplements having indices above or below 100 were proportionately more or less effective than the original acid precipitate, both being fed in equivalent quantities with a few exceptions, which are noted in Table I.

As reported previously (1), the growth factor was stable to autoclaving at neutral pH. It was destroyed by autoclaving with 2 N HCl. It was not destroyed by autoclaving 2 hours at a pH of 8.5, since that portion of the solution which was acidified to pH 3.0 after autoclaving yielded a potent precipitate. The remainder of the solution was added to the diet without adjusting the pH, since the pH had decreased from 8.5 to 7.6 during autoclaving. The activity disappeared from this part of the solution, presumably after mixing with the diet or while it was held at pH 7.6 before mixing. The active factor was still precipitated at pH 3.0 after autoclaving at pH 8.5 or 7.0.

Precipitation of inert material by half saturation with  $(\text{NH}_4)_2\text{SO}_4$  proved to be an unsatisfactory procedure because the results were not readily reproducible. The first three times it was tried the greater part of the potency was in the soluble fraction, but in later experiments most of the active material, and in one case all of it, was precipitated. Precipitation at 43 or 33 per cent saturation with  $(\text{NH}_4)_2\text{SO}_4$  did not offer any greater promise. The active material as it occurred in the  $(\text{NH}_4)_2\text{SO}_4$  solubles was not precipitated at acid pH (Preparation 3); as it occurred in the redissolved  $(\text{NH}_4)_2\text{SO}_4$  precipitate, it was precipitated by acid (Preparation 6). It seems likely that the factor was associated with protein in the  $(\text{NH}_4)_2\text{SO}_4$  precipitate, but that it was free of such association in the solubles. The factor in its  $(\text{NH}_4)_2\text{SO}_4$ -soluble, acid-soluble state was apparently non-dialyzable (Preparation 3).

Like the  $(\text{NH}_4)_2\text{SO}_4$  procedure, precipitation with 2 per cent  $\text{CaCl}_2$  removed much inert material, while leaving a considerable portion of the active factor in solution. However, removal of the calcium as  $\text{CaCO}_3$  removed varying quantities of the activity, and in Preparation 5 the calcium-free solubles were devoid of activity. The active factor in the  $\text{CaCl}_2$  solubles as in the  $(\text{NH}_4)_2\text{SO}_4$  solubles was not precipitated at acid pH.

When acetone to the extent of 80 per cent concentration was added to solutions of the growth factor, activity was divided between the solution and the precipitate, but if the precipitate was redissolved and reprecipitated, the second precipitate contained little of the factor, most of it being in the two soluble fractions. The factor as it occurred in 80 per cent acetone was partially soluble and partially precipitable at pH 3.0.

After digestion of the acid precipitate with papain the factor was not precipitable at pH 3.0. This confirms the supposition stated above that precipitation of the factor by acid was the result of association with protein. A precipitate containing relatively little of the factor formed in the soluble fraction at pH 3.0 when this was neutralized and heated to boiling. This precipitate is believed to have been derived largely from the papain preparation. The factor present in the papain digest apparently was incompletely adsorbed by norit, and that which was adsorbed was incompletely eluted by 10 per cent  $\text{NH}_4\text{OH}$ . In view of the destruction of the factor after autoclaving at pH 8.5, it is also possible that some potency may have been lost from the eluate as it was boiled to remove  $\text{NH}_3$ . This was done at atmospheric pressure.

The factor was readily extracted from the acid precipitate by ammoniacal ethanol but not by neutral or acid ethanol. After extracting with neutral ethanol at 65–70° to remove some inert material, the residue did not yield its content of the factor as readily to ammoniacal ethanol as did the original precipitate. The results obtained with Preparation 4 are similar to those obtained after autoclaving at pH 8.5, in that the ammoniacal ethanol extract was inactive when fed as such but showed activity in both fractions when it was adjusted to pH 3.0 and separated into the precipitate and the solution. These results suggest further investigation of the lability of the active material in weakly alkaline solutions and especially during the drying of such solutions.

The previously reported precipitation of the active material at pH 3.0 was undoubtedly due to the presence of protein, since digestion with papain left the factor no longer precipitable at this pH. The precipitation of the factor with protein at pH 3.0 was probably due to occlusion or coprecipitation rather than to chemical combination of the factor with the protein molecule, because in 2 per cent  $\text{CaCl}_2$  solution and in several cases in half saturated  $(\text{NH}_4)_2\text{SO}_4$  solution the protein precipitated and left the active factor in solution in a form no longer precipitable at pH 3.0.

McGinnis *et al.* (6) reported that an ethanol-soluble fraction of liver contained a factor essential to the growth of chickens fed a basal diet similar to the diets used in these studies. This factor was dialyzable, soluble in water at pH 3.0, and not adsorbed by activated charcoal at pH values from 2.0 to 5.0. The properties reported by McGinnis and those reported in this paper are in good agreement except for the results of dialysis experiments, and since these were probably carried out under different conditions and certainly with different preparations they would not exclude the possibility that a single factor was involved in both studies.

## SUMMARY

The essential dietary factor for chickens previously reported to be present in concentrates prepared from cow manure was soluble in water at pH 3.0 if the protein was previously removed by digestion with papain, or by precipitation from half saturated  $(\text{NH}_4)_2\text{SO}_4$  solution or 2 per cent  $\text{CaCl}_2$  solution. The factor was soluble in 80 per cent acetone. It was extracted from the crude acid precipitate containing protein and other inert material, to a slight extent by neutral ethanol, and completely by ammoniacal ethanol. It was stable when autoclaved 2 hours at neutral pH but readily destroyed by autoclaving 1 hour with 2 N acid. There was some evidence of destruction of the factor when it was allowed to stand in slightly alkaline solutions or when such solutions were dried.

A method of assaying preparations for their content of the factor by a growth test of 1 or 2 weeks duration was developed.

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## THE HYALURONIDASE INHIBITOR OF HUMAN BLOOD

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The disappearance of the "spreading factor" from the blood stream after intravenous injection was demonstrated by Duran-Reynals. He suggested the presence of a substance in blood which destroys the "spreading factor" (1). Hobby *et al.* (2) found evidence of the inhibition of hyaluronidase prepared from *Clostridium welchii* and certain strains of streptococci by normal human and rabbit serum. This effect was ascribed to salt formation between albumin and hyaluronic acid.

McClean (3) reported that hyaluronidases prepared from bull, rabbit, and mouse testes were inhibited by guinea pig, rabbit, sheep, horse, mouse, and human serum. Heparin, chondroitin sulfate, and gastric mucin were found to be inhibitory, while Shiga-Kruse polysaccharide and blood group A hapten were without activity. The inhibitor in blood was not considered identical with any of these substances, since chemical properties indicated that it was pseudoglobulin in nature.

A recent series of papers by Haas (4-6) has revived interest in the nature of the hyaluronidase inhibitor in blood. On the basis of reaction rates and effects of temperature changes it was concluded that the substance in blood is an enzyme and the name "antinvasin I" was suggested. Comparison of the relative activity of blood sera from different species on different hyaluronidases led to the conclusion that there exists a complex system consisting of at least two different antinvasins and a substance accompanying hyaluronidase which was named "proinvasin." Haas suggests that this complex system is responsible for defense against bacterial invasion. This work was done with crude hyaluronidases and no account was taken of the possible effects of contaminating enzymes.

In a preliminary report (7) from this laboratory evidence was presented casting doubt on the enzyme nature of the substance in blood which interferes with hyaluronidase activity. More recently Hechter (8) has studied the effect of serum on the spreading activity of hyaluronidase and found evidence of inhibition. He quotes unpublished work by Hiddian and Pirie as proving that the serum factor acts as an inhibitor rather than an enzyme.

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Leonard and Kurzrok have shown that serum also inhibits the dispersal by hyaluronidase of follicle cells surrounding the ovum (9).

The effects discussed above are all presumably due to a physiological substance in blood serum. This should be distinguished from the specific antibodies for individual hyaluronidases which have been repeatedly demonstrated (10-13).

It is the purpose of this report to present evidence showing that the behavior of the substance in blood is inconsistent with the thesis that it is an enzyme. A relatively simple method has been devised for the estimation of this substance and some success has been achieved in its purification. All studies have been concerned only with inhibition of bovine hyaluronidase by human serum.

### *Methods*

The viscosity method of Madinaveitia and Quibell (14) was used as modified by Haas (4). Due to the tedious nature of this method it was used only for comparative purposes and was finally abandoned in favor of a simpler turbidity method. Materials used were prepared as previously described (15).

Before examining results obtained by the viscosity method it is necessary to consider the method of calculation of activity. If the time required to reach a half reduction of viscosity of a given amount of hyaluronic acid in the presence of hyaluronidase (half life time) is called  $R_0$ , the activity of hyaluronidase may be expressed as the reciprocal of the half life time ( $1/R_0$ ). If the same amount of hyaluronidase is mixed with a given amount of serum, a new half life time is obtained which may be designated as  $R$ . The activity of hyaluronidase remaining after the action of serum can be expressed then as  $1/R$  and the amount of hyaluronidase that is destroyed (or inhibited) is  $1/R_0 - 1/R$ . Haas has used this method of computation in some experiments but in others he has used the expression  $(R - R_0)/R_0$ . The derivation and meaning of this expression are not given. It is not equal to the previous expression ( $1/R_0 - 1/R$ ) and has no meaning when methods of hyaluronidase assay not involving the half life time are used. Using the method as described by Haas, we have been unable to find any linear relationship between activity and amount of serum. If, however, activity is expressed in terms of amount of hyaluronidase activity destroyed ( $1/R_0 - 1/R$ ), linearity is obtained. Under these conditions the results obtained by the turbidity method do not differ significantly from those by the viscosity method.

The turbidity method used is essentially that described in a previous publication from this laboratory (15). A standard curve for hyaluronidase is run daily to check on reagents and methods. It has been previously pointed out that the activity of hyaluronidase varies with different prepa-

rations of hyaluronic acid. In order to obviate this difficulty we have defined our unit in terms of the activity of a given amount of a standard hyaluronidase preparation. No evidence of change has been found in 10 months storage in the dry state at  $-20^{\circ}$ .

To determine inhibitor in serum the following method is used. Standard enzyme is diluted in 0.1 M borate buffer at pH 7.4 so as to contain 24 units per ml. 0.5 ml. of this solution is mixed with 0.5 ml. of diluted serum (serum is diluted with 0.15 M NaCl to appropriate concentration, depending on activity) and the mixture is incubated for 10 minutes at  $24^{\circ}$ . At the end of this time the mixture is brought to  $38^{\circ}$  and 1 ml. of hyaluronic acid made up in 0.3 M phosphate buffer at pH 5.5 is added. This mixture is incubated at  $38^{\circ}$  for 45 minutes, after which time the turbidity is de-

TABLE I  
*Comparison of Activity of Sera As Determined by Viscosity and Turbidity Methods*

Sample No.	Viscosity		Turbidity, units per ml.
	$\frac{1}{R_0} - \frac{1}{R}$ per ml.	$\frac{R - R_0}{R_0}$ per ml.	
1	86	46	83
2	58	19	56
3	51	29	51
4	45	11	35
5	32	7	32
6	92	64	72

veloped and read as previously described. Turbidity developed due to the addition of the serum is corrected for by a blank containing all reagents except hyaluronidase. The activity of serum is determined in terms of amount of enzyme which has been inhibited. 1 unit of inhibitor is that amount which will inhibit 1 unit of hyaluronidase. Results are expressed in terms of units of inhibitor per ml. of serum. Table I shows the results of assay of six sera by both the viscosity and turbidity methods. With the methods as outlined above the results obtained are comparable to those obtained by the viscosity method, provided calculations are made by the formula  $1/R_0 - 1/R$ . If the formula  $(R - R_0)/R_0$  is used, this is not true. It will be noted that not only do the absolute values differ but the relative values on different sera do not agree.

Since good agreement between the turbidity and viscosity methods was obtained, the turbidity method was used in the remaining studies.

#### EXPERIMENTAL

*Relationship of Activity to Concentration of Serum*—It has previously been demonstrated by the viscosity method that the activity of serum varies

linearly with serum concentration (4). In this case activity was expressed as a function of hyaluronidase destroyed. Similar experiments by the turbidity method have confirmed this finding. The results of such an experiment are shown in Fig. 1. The curve shown is the calculated regression line by the method of Fisher (16) from the observed points up to and including 0.05 ml. of serum. It is obvious that above this point the curve shows flattening. For this reason, values were accepted as valid only if destruction did not exceed 4 units (in the presence of 6 units) of hyaluronidase. If the amount of enzyme inhibited falls below 2 units, the intrinsic error of the turbidity method becomes very large. This is illustrated by

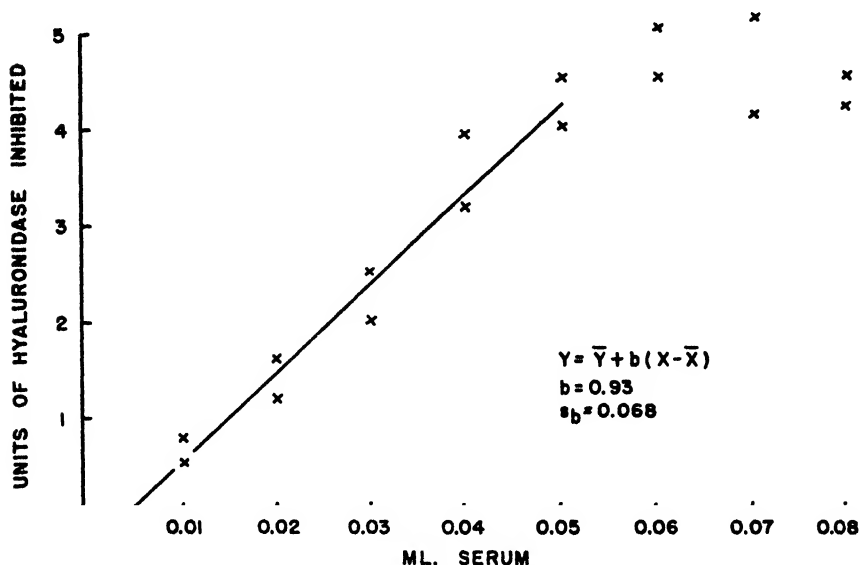


Fig. 1. Relationship of hyaluronidase inhibitor activity to serum concentration.

the following example. If 6 units of hyaluronidase are added and 1 unit is inhibited by a given amount of serum, 5 units will remain. In a previous publication it has been shown that at this level the coefficient of variation in the turbidity determination is about 5 per cent; thus the value of 5 units may be considered as  $5 \pm 0.25$ . Since the inhibitor value is obtained by difference from 6, its value will be  $1.0 \pm 0.25$  unit; thus the error in the inhibitor becomes  $\pm 25$  per cent, although the actual experimental error in the determination is only  $\pm 5$  per cent. By this same type of calculation it can be shown that with between 2 and 4 units of inhibitor the coefficient of variation should vary between 5 and 20 per cent. In a series of 300 determinations done under these conditions the coefficient of variation was found to be empirically  $\pm 14$  per cent.

*Effect of Hyaluronidase Concentration on Activity of Serum*—It has previously been claimed that the activity of serum is directly proportional to hyaluronidase concentration (4). This is to be expected whether the substance in serum is an inhibitor or an enzyme, provided the amount of hyaluronidase is low. If the concentration of hyaluronidase is in excess of

TABLE II  
*Effect of Hyaluronidase Concentration on Activity of Serum*

Hyaluronidase present, units .....	6.0	8.0	10.0
“ inactivated, units .....	3.1	3.2	3.1

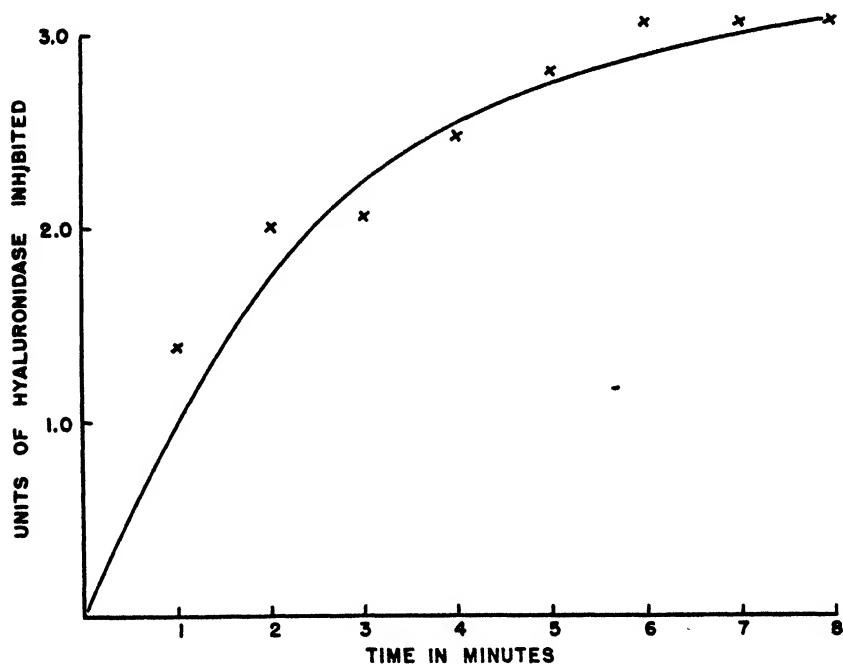


FIG. 2. Rate of reaction between serum and hyaluronidase

that required for maximum activity, the activity of the serum should become independent of hyaluronidase concentration. That this is so is shown by the data in Table II.

*Effect of Time on Reaction between Hyaluronidase and Serum*—The next group of experiments was performed in order to determine the effect of time on the activity of blood serum. This is one of the criteria upon which Haas has concluded that the substance in question is an enzyme. The results of such an experiment are shown in Fig. 2. It will be noted that the

reaction is apparently complete in 5 minutes, despite the fact that only 50 per cent of the hyaluronidase present has been inhibited. When Haas' data (4) are recalculated by the formula  $(1/R_0 - 1/R)$ , his results are found to agree well with those in Fig. 2.

The findings in this experiment are inconsistent with the assumption that the substance in question is an enzyme.

*Effect of Temperature on Activity of Serum*—The next experiments were performed to study the effect of temperature on the rate of reaction between serum and bovine testicular hyaluronidase. Table III illustrates the results of a group of such experiments. The serum and enzyme were mixed and kept at the indicated temperature. After 10 minutes hyaluronic acid dissolved in 0.3 M phosphate buffer was added and the mixture brought to 38° for the determination of the remaining hyaluronidase. It will be noted that higher activity was consistently obtained at lower temperatures.

TABLE III  
*Effect of Temperature on Reaction of Serum and Hyaluronidase*

Sample No.	7°	17°	27°	38°
	Units per ml. serum			
1	79	76	66	47
2	100	100	71	51
3	94	92	79	48

With some purified preparations (prepared according to the method of Hahn (17)) no difference between different temperatures was obtained.

The activities at these temperatures are at variance with those previously reported (4) and are again inconsistent with the assumption that the active substance in blood is an enzyme.

The decrease in activity at higher temperatures might be due to the presence of contaminating enzymes in the hyaluronidase preparations, which destroy the inhibitory substance in serum. That this is true is suggested by the fact that no change with temperature was obtained with some purified preparations.

Such contaminating enzymes may be responsible for the effects described by Haas as due to proinvasins. It seems unnecessary to postulate a complex scheme for the explanation of these effects.

*Effect of pH and Salt Concentration on Activity of Serum*—Studies on the effect of pH and salt concentration on the reaction between serum and hyaluronidase were made difficult by the high buffer capacity of serum. Marked inhibition of activity was found at an ionic strength above 0.2, while if the ionic strength was below 0.1, difficulty was encountered in

maintaining a constant pH with different amounts of serum. If a 0.1 M borate buffer at pH 7.4 was used, this difficulty was circumvented.

Table IV shows the variation in serum activity with variation of pH at constant ionic strength ( $\mu = 0.12$ ). In general these results confirm those previously reported.

The inhibition of the reaction by phosphate and sulfate ions has been confirmed. The effect of sulfate is of practical importance, since the purification of hyaluronidase usually involves ammonium sulfate purification. Care must be taken to remove sulfates completely prior to use of hyaluronidase for determination of inhibition by serum.

TABLE IV  
*Effect of pH on Activity of Serum*

Serum No.	pH 5.8	pH 6.6	pH 7.6	pH 8.3
	Units per ml. serum			
1	0	53	58	50
2	0	34	46	40

TABLE V  
*Variation of Serum Hyaluronidase Inhibitor with Age and Sex*

The results are expressed in units per ml.

	Males				Females	
Age, yrs.	0-15	16-30	31-45	>45	16-30	31-45
No. of individuals	7	32	8	7	10	13
" " determinations	17	53	11	12	25	26
Mean	126.7	85.1	89.1	149.4	112.4	117.6
Standard deviation	39.8	34.6	35.4	58.6	42.6	32.8

### *Chemical Properties*

*Stability*—As previously pointed out, the substance in blood which inhibits hyaluronidase is quite unstable. At 50° the activity is completely destroyed in 10 minutes at pH 7.4. Even at 4° at this pH there is about 25 per cent destruction in 48 hours. Some variation has been found in different sera, thus making it difficult to draw final conclusions as to the stability at lower temperatures.

All sera tested were stable at -20°, some having been kept for as long as 6 months with no detectable loss of activity.

*Purification*—The inhibitor is apparently of large molecular weight, since it does not dialyze. It can be separated from serum by Method 6 of Cohn *et al.* (18) and the activity can be quantitatively recovered in Fractions II and III.



All of the activity is found in the plasma. No difference between the activity of serum and plasma has been found.

#### *Distribution in Normal Human Serum*

Nothing is known regarding the distribution of this substance in normal or pathological states. Table V gives the results from a number of normal individuals divided by age and sex. By Fisher's *t* test it is found that males of reproductive age (15 to 45) have a significantly lower range than any other group studied. An insufficient number of female children or females over 45 years have so far been studied from which to draw definite conclusions regarding these groups. The wide variations within the normal group remain to be explained. Preliminary evidence indicates that the inhibitor level in the serum of males is inversely related to the hyaluronidase concentration of semen.

#### DISCUSSION

There seems to be little question that there exists in the blood of a number of species of animals an inhibitor of the enzyme hyaluronidase. This substance is apparently quite distinct from specific antibodies to particular hyaluronidases. The question of specificity of the inhibitor for particular hyaluronidases remains for future work, since a final answer can only be obtained when pure hyaluronidases are available. The work reported here has been concerned only with the reaction between bovine testicular hyaluronidase and human serum.

The evidence presented in this paper lends no support to the thesis that the substance in blood is an enzyme. The findings are consistent with the idea that this substance is an inhibitor. McClean (3) has shown that a number of polysaccharides act as inhibitors of hyaluronidase, and the suggestion has been made that this substance in blood is a competitive inhibitor (8). Since present evidence indicates that the inhibitor in blood is protein in nature, it is unlikely that any of the carbohydrates used by McClean are responsible for the inhibition in serum. It is possible, however, that it may have a polysaccharide prosthetic group which permits it to act as a competitive inhibitor.

The significance of variations in distribution of this substance remains to be determined. The relationships to sex and age together with the known rôle of hyaluronidase in fertilization suggest that this enzyme system is probably under endocrine control. Elucidation of these mechanisms may serve to further our understanding of the mechanism of action of certain hormones.

## SUMMARY

1. A simple and rapid method has been devised for the estimation of hyaluronidase inhibitor in human blood.

2. Kinetic studies on the inhibition of hyaluronidase of bovine testes indicate that this substance is an inhibitor rather than an enzyme, as previously claimed.

3. Chemical studies suggest that this substance is protein in nature.

4. Studies of blood levels in normal individuals show a lower level in males of reproductive age than in other groups studied.

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# THE VITAMIN A CONTENT OF FETAL RATS FROM MOTHERS ON A HIGH CHOLESTEROL DIET\*

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The concentration of vitamin A in fetal liver and plasma has been reported to be considerably lower than that found in maternal liver and plasma (1-5). Although various factors may contribute to this maternal-fetal difference, a placental mechanism which limits the movement of vitamin A to the fetus seems to be involved. Lund and Kimble (4) have suggested that the passage of vitamin A to the fetus may be related to, or limited by, the lipide metabolism of the placenta. More recently, it has been shown that the lipide concentration in the placenta influences the movement of maternal nutrients to the fetus (6).

It seemed possible, therefore, that an alteration in the lipide metabolism of the placenta might affect the transplacental movement of vitamin A. For this reason, the rôle of lipides in the placental transmission of vitamin A was studied, with the technique by which Popják (6) had shown that the feeding of a high cholesterol diet will cause a large deposition of cholesterol esters and some excess of neutral fat over normal in the placenta. In the present experiments, an attempt was made to determine the effect of a high concentration of lipide in the placenta on the movement of vitamin A from the mother to the fetus.<sup>1</sup>

## EXPERIMENTAL

In order to maintain the control and experimental animals at approximately the same level of vitamin A nutrition, weanling female rats, to be used eventually as maternal animals, were kept on a vitamin A-free diet until the growth curve began to level off. From this time onward, small

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<sup>1</sup> The question of the transport of vitamin A across the placenta became of interest to us in connection with the study of congenital eye lesions. It has been shown that pregnant animals, severely deficient in vitamin A, will produce fetuses with malformations of the eye which resemble the fibroplasia found behind the lenses of some premature infants (7-9). However, the deficiency required in the experimental animals is so severe that the fetuses are usually resorbed and only rarely born alive (7, 8). If the placental permeability could be depressed, the fetuses might be essentially deprived of vitamin A, while the maternal animals would still contain enough vitamin A to complete gestation successfully.

oral supplements of vitamin A were given until the animals reached a weight of about 200 gm.; they were then bred. The vitamin A supplements were continued until the end of the experiment.

In Experiment I, the vitamin A supplements were begun about 6 weeks before breeding. A total of 325 I.U. of vitamin A was given in 63 days. 2 days before mating, these rats were divided into two groups. The control group was continued on the basal diet; the experimental animals were fed a 2.5 per cent cholesterol diet, prepared by substituting reprecipitated cholesterol (6) for an equal weight of sucrose in the basal diet. In Experiment II, the same procedure was followed, except that a total of 95 I.U. of vitamin A was given in 50 days, and the rats were divided into a control group and one receiving 5 per cent cholesterol before breeding. Another experiment was attempted in which the maternal rats were fed an 8 per cent cholesterol diet. The animals on this diet resorbed their fetuses when gestation was about half completed.

The basal diet consisted of 68 gm. of sucrose, 18 gm. of vitamin-test casein (Smaco), 10 gm. of corn oil, 4 gm. of salt mixture (10), 0.8 mg. of thiamine, 0.8 mg. of riboflavin, 0.8 mg. of pyridoxine, 1.0 mg. of calcium pantothenate, 10 mg. of nicotinamide, and 10 mg. of inositol. In addition, all the animals received a weekly supplement of 50 I.U. of vitamin D<sub>2</sub>, 1.2 mg. of  $\alpha$ -tocopherol, and 7.5  $\gamma$  of 2-methylnaphthoquinone.

Shortly before the completion of gestation, blood samples were taken from the maternal rats by heart puncture. They were then killed and the fetuses removed from the uterus. Maternal liver samples were also taken. All the tissue samples, and the plasma obtained, were frozen at once and kept in the ice box ( $-4^{\circ}$ ) until they were analyzed for their vitamin A content (usually 1 to 3 weeks). In Experiment I, two litters were obtained within 2 hours after birth. In no instance was milk found in the stomachs of the young.

The plasma vitamin A was determined by the spectrophotometric micromethod of Bessey *et al.* (11). The whole fetuses, the liver samples, and a number of placentae were analyzed for their vitamin A content by the method previously used (12). The tissue samples were digested in 5 to 10 volumes of alcoholic potassium hydroxide (3.5 N in 60 per cent ethanol) on a hot water bath for 30 minutes, and then extracted with a 1:1 mixture of xylene and kerosene. The absorption of the vitamin A in the extract was measured in a Beckman spectrophotometer, before and after ultraviolet irradiation, and compared with a standard curve of the absorption of known amounts of crystalline vitamin A alcohol, similarly treated.

The placental fat was determined from an ether extraction of an acidified, alkaline hydrolysate of the tissue. Although only the fatty acids and the

non-saponifiable fractions of the total fat are measured by this procedure, it is adequate for comparative purposes.

### RESULTS AND DISCUSSION

The ether-extractable lipid fraction from the hydrolyzed placental tissue showed markedly divergent values for the control and the cholesterol-

TABLE I  
*Distribution of Vitamin A in Maternal Rats*

Experi- ment No.	No. of ma- ternal rats	No. of fetal rats	Diet	Weight	Plasma vi- tamin A	Liver vi- tamin A
				gm.	I.U. per cent	I.U. per gm.
I	6	36	Control	245	86	19.1
	6	44	2.5% cholesterol diet	246	93	21.6
II	9	65	Control	192	45	6.6
	6	38	5% cholesterol diet	194	41	6.9

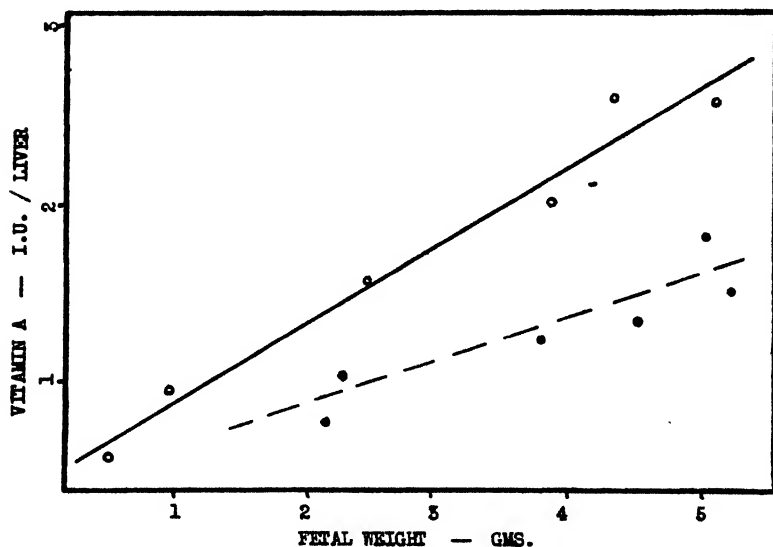


FIG. 1. The effect of feeding a 2.5 per cent cholesterol diet to maternal rats on the vitamin A content of the fetal livers. Each point represents the average results from one litter. ○ control fetal livers, ● experimental fetal livers.

fed rats. The control placentae contained an average of 1.85 per cent of ether-extractable material, while the placentae from the experimental rats contained 4.53 per cent on the basis of fresh tissue.

The distribution of vitamin A in the maternal animals is shown in Table I. It can be seen that the control and the corresponding experimental

maternal animals contained approximately the same amount of vitamin A. Presumably, both groups in each experiment could supply their fetuses with about the same amount of vitamin A, and therefore the differences in the vitamin A content of the fetuses can be attributed to a difference in vitamin A transmission through the placentae.

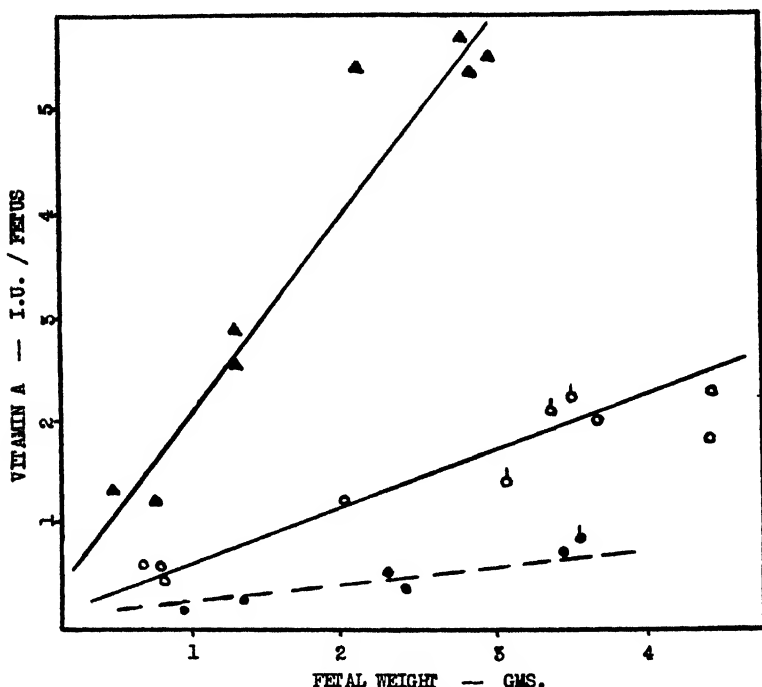


FIG. 2. The effect of feeding a 5 per cent cholesterol diet to maternal rats on the vitamin A content of the fetuses. ▲ fetuses from mothers on a normal stock diet, ○ fetuses from control rats, and ● experimental fetuses. The points on the graph shown with a | have been computed from the analyses of fetal livers.

In Experiment I, the control fetal livers contained about 50 per cent more vitamin A per gm. than was found in the livers of the fetuses from the cholesterol-fed mothers. The vitamin A content of the fetal livers as a function of fetal weight is presented in Fig. 1. A comparison of the results indicates that vitamin A accumulates more rapidly in the control fetal livers.

Fig. 2 shows the results of the analyses of whole fetuses obtained in Experiment II. For comparison, the vitamin A content of whole fetuses from mothers who were kept on a normal stock diet is included. The fetuses from the cholesterol-fed mothers contained significantly less vitamin

A than those from the control mothers. Four of the values plotted in Fig. 2 were computed from the analyses of fetal livers.

Okey (13, 14) has indicated that large amounts of fat and cholesterol are deposited in the livers of cholesterol-fed guinea pigs. The livers of the cholesterol-fed maternal rats in our experiments showed the typical light color associated with fatty deposits in the liver. The control maternal livers appeared to be normal. There was no grossly observable difference in the fetal livers from the control or experimental animals.

Because of the low vitamin A content, and the small amount of tissue available, the results of the vitamin A analyses of the placentae were not considered highly reliable. However, there was no appreciable difference in the range of values for the control and the experimental placentae.

The preceding data demonstrate that an inverse relationship exists between the lipide content of the placenta and the movement of vitamin A across it. It has been reported that vitamin A in the plasma does not behave as if it were either in a colloidal state or dissolved in a fatty solute, but seems to be associated with some plasma protein in a "carrier complex" (15, 16). The excess placental lipide may then depress the transplacental movement of vitamin A either by blocking a mechanism for splitting the "carrier complex" or by decreasing the already low permeability of the placenta to large molecules.

I wish to express my thanks to Miss Muriel Appel for technical assistance in the prosecution of this study.

#### SUMMARY

1. Two groups of female rats were brought to approximately the same nutritional level with respect to vitamin A. The control group was fed a basal diet; the other rats were fed the same diet with either 2.5 per cent or 5 per cent of cholesterol added. The animals were mated. Shortly before parturition, the maternal animals were killed and vitamin A was determined in the fetuses, fetal livers, and maternal livers and plasma.

2. The placentae of the cholesterol-fed mothers were found to contain about twice as much lipide as those from control mothers.

3. The whole fetuses and the fetal livers from the cholesterol-fed mothers contained significantly less vitamin A than those from the control mothers. It is concluded that the fatty placentae inhibit the movement of vitamin A from the maternal circulation to the fetus.

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# THE UTILIZATION OF D-TYROSINE FOR GROWTH IN THE RAT\*

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The conversion of phenylalanine to tyrosine in the animal body has been established by the growth experiments of Womack and Rose (1) and the isotopic studies of Moss and Schoenheimer (2). However, the reversibility of this reaction probably does not occur.

Tyrosine, usually considered as a non-essential amino acid for the growth of the white rat, has been shown by Womack and Rose (3) to be able to replace about one-half of the phenylalanine requirement of the growing rat. This indicates that phenylalanine may be converted to meet certain demands of the organism for tyrosine, since this requirement can be met equally well by either phenylalanine or tyrosine.

Both enantiomorphs of the amino acid can be utilized for growth in the rat in the case of tryptophan (4), phenylalanine (5), methionine (6), and histidine (7), while the unnatural isomer fails to show growth responses in the case of valine, leucine, isoleucine (8), threonine (9), and lysine (10). Since tyrosine is non-essential in the presence of sufficient phenylalanine, the growth effect of D-tyrosine has never been established. The observations of Womack and Rose (3) now make it possible to determine the availability of D-tyrosine for growth in the rat.

Using human subjects, Albanese, Irby, and Lein (11), employing an excretion technique, report that sufficient tyrosine and aliphatic organic acids are found in the urine, following the ingestion of 0.01 mole of DL-tyrosine, to account for nearly all of the D isomer in the racemic mixture. The conclusion drawn from the data is that the D isomer is poorly or totally unavailable for normal physiological functions in man. This is further substantiated by the very slight excretion of tyrosine and organic acids when 0.01 mole of L-tyrosine is fed. However, there is no direct evidence

\* The data in this paper are taken from a thesis presented by Edward C. Bubl to the Faculty of the Graduate School of Oregon State College in partial fulfillment of the degree of Doctor of Philosophy.

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We are indebted to Merck and Company, Inc., Rahway, New Jersey, for a generous supply of the amino acids used in this study.

presented which would identify the urinary tyrosine as coming from the D component.

The following study was undertaken to determine the growth responses to D-tyrosine when fed to rats in an amino acid mixture with suboptimal amounts of phenylalanine.

TABLE I  
*Composition of Amino Acid Mixture*

	gm.
Glycine.....	0.1
Alanine.....	0.7*
Serine.....	0.2*
Valine.....	2.0*
Leucine.....	2.4*
Isoleucine.....	1.6*
Cystine.....	0.2
Methionine.....	0.8*
Threonine.....	1.4*
Tryptophan.....	0.4*
Aspartic acid.....	0.2
Glutamic ".....	2.0
Lysine monohydrochloride.....	3.0*
Proline.....	0.2
Histidine monohydrochloride.....	0.95
Arginine.....	0.60
Sodium bicarbonate.....	1.76
	18.51
Tyrosine.....	0.5
Phenylalanine.....	0.5*
	19.51

\* Denotes racemic acid.

#### EXPERIMENTAL

Three litters of Evans-Long strain rats were used in this study. They were distributed as evenly as possible with respect to weights, litters, and sex. At 28 days of age the animals were placed in individual cages and were allowed water and the experimental diet *ad libitum*. Weights were recorded daily.

The basal diet and the vitamin supplements were made up according to Borman *et al.* (12), with the exception that biotin was omitted. It was felt that the liver extract would supply this requirement or at least that this would not be a limiting factor in a 28 day experiment. The animals

were fed daily by placing a weighed amount of food in the containers. The food thus measured was always in excess of what might be eaten. The food intakes were calculated by difference after careful collection of any spillage.

The amino acid mixture was prepared from crystalline amino acids, some of which were either synthesized or isolated in our laboratory, while

TABLE II

*Experiments Showing Weight Gain with Phenylalanine and Tyrosine Added to Diet*  
Each experiment covered 28 days.

Litter No.	Rat No. and sex	Total gain in weight	Total food intake	Amino acid supplements
		gm.	gm.	
6	34 ♀	45	182	
6	35 ♀	42	167	
6	36 ♀	26	170	0.5% DL-phenylalanine
7	86 ♂	47	188	0.5% DL-tyrosine
8	87 ♂	43	216	
8	88 ♂	53 (42.6)*	223	
6	37 ♀	46	177	
6	38 ♂	47	199	0.5% DL-phenylalanine
6	39 ♂	43	188	0.5% L-tyrosine
7	85 ♀	32 (42.0)*	196	
7	81 ♂	57	238	
7	82 ♂	25	202	0.5% DL-phenylalanine
7	83 ♀	39	216	0.5% D-tyrosine
7	84 ♀	59 (45.0)*	232	
6	40 ♂	24	194	0.5% DL-phenylalanine
6	41 ♂	36 (30.0)*	173	0.25% L-tyrosine
6	42 ♀	Died after 2 wks.		0.25% DL-alanine

\* Average for group.

some were from commercial sources. In every case these compounds gave excellent analytical values, indicating a high degree of purity.

The amino acid mixture was patterned after Borman *et al.* (12), except that phenylalanine and tyrosine were omitted and arginine was added. Due to the difficulty of obtaining a satisfactory sample of hydroxyproline, this amino acid was omitted. Since this is probably a dispensable amino acid (13), it was felt that this omission was not critical. The alanine content was increased slightly to compensate for any lowered nitrogen content.

The DL-tyrosine was prepared by racemization of L-tyrosine, according to the method of du Vigneaud and Meyer (14).

*Analysis*— $C_9H_{11}O_2N$

Calculated. N (Kjeldahl) 7.73, N (Van Slyke) 7.73

Found. " " 7.70, " " " 7.75

$[\alpha]_D^{25} = 0.00^\circ$  (4% tyrosine in 4% HCl)

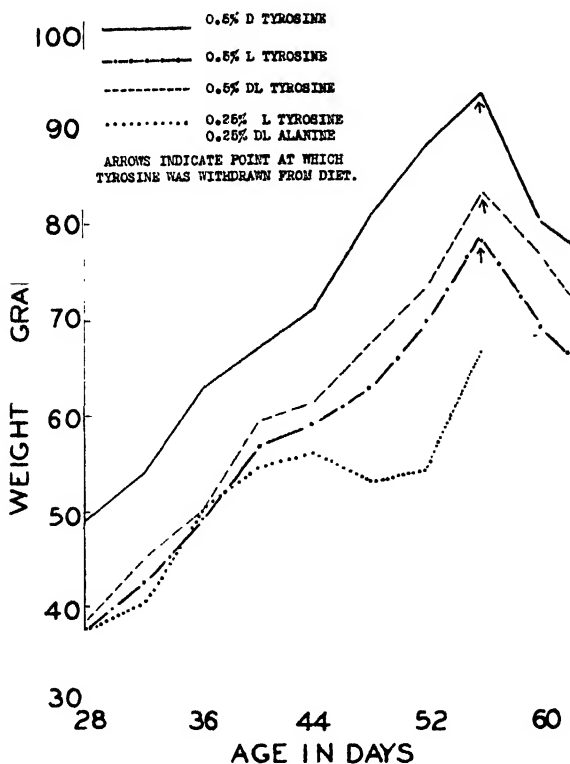


FIG. 1. Growth response of 28 day-old rats to a basal diet containing 0.5 per cent phenylalanine supplemented with DL- or DL-tyrosine.

The DL-tyrosine was resolved with brucine, as reported by Sealock (15). The D isomer gave the following analytical values:

*Analysis*— $C_9H_{11}O_2N$

Calculated. N (Kjeldahl) 7.73, N (Van Slyke) 7.73, C 59.66, H 6.12

Found. " " 7.75, " " " 7.77, " 59.62, " 6.16

$[\alpha]_D^{25} = +9.5^\circ$  (4% tyrosine in 1.0 N HCl)

#### DISCUSSION

The demonstration by Womack and Rose (3) that L-tyrosine stimulates growth when phenylalanine is furnished in suboptimal amounts makes available a method for testing the nutritional availability of the unnatural isomer of tyrosine. These workers have shown that 0.4 per cent DL-

phenylalanine, when added to the purified amino acid mixture, did not allow the animals to grow at a good rate; however, upon the inclusion of increasing levels of L-tyrosine the growth was improved. The addition of 0.5 per cent L-tyrosine seemed to give the best growth.

In Table II the weight gain of rats receiving 0.5 per cent of phenylalanine and an additional 0.5 per cent of either D-, L-, or DL-tyrosine is shown. It will be seen that feeding the L isomer confirms very beautifully the report of Womack and Rose (3). Also, there is no significant difference in weight gain when like amounts of D- or DL-tyrosine are substituted. In a second series of experiments in which the L-tyrosine was lowered to 0.25 per cent, the growth of two rats was markedly inferior, while the third died at the end of 2 weeks. It might be pointed out that at this level the L-tyrosine in the mixture would be the same as in the diet containing 0.5 per cent DL-tyrosine. If the D isomer had no retarding effect and were not available for growth, the response in each of these experiments should be equal. This is not so, since the growth was superior on the DL-tyrosine diet, thus giving a strong support to the idea that the D isomer is nutritionally available. This, of course, is also indicated by the experiments when D-tyrosine, devoid of any of the L component, is fed.

Fig. 1 shows the effect of withdrawing the 0.5 per cent tyrosine, but allowing 0.5 per cent phenylalanine to remain in the diet. One rat from each group was continued for the 6 day period. In all three experiments the response was the same; namely, the loss of 10 to 13 gm. during a 6 day period.

We wish to express our appreciation to Mr. S. C. Fang for some of the combustion analyses reported in this paper.

#### SUMMARY

1. When suboptimal amounts of phenylalanine are incorporated in an amino acid mixture otherwise adequate for the white rat, the additional requirement is met equally well with D-, L-, or DL-tyrosine.

2. If one may conclude from the indirect evidence (11) that the human is unable to utilize D-tyrosine, then there must exist a species difference in the use of this enantiomorph.

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# THE OXIDATIVE DEMETHYLATION OF MONOMETHYL-L-AMINO ACIDS

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(Received for publication, March 9, 1948)

Handler, Bernheim, and Klein (1) have shown that the oxidation of the N-methyl derivatives of DL-methionine, DL-leucine, DL-alanine, and DL-histidine by rat liver and kidney preparations is due to the presence of D-amino acid oxidase, and that the N-methyl derivatives of DL-phenylalanine, DL-tryptophan, and DL-valine are not oxidized by these preparations.

Blanchard, Green, Nocito, and Ratner (2) have found that L-amino acid oxidase obtained from rat kidney and liver catalyze the oxidation of the N-methylamino acids, viz. those of L-leucine, methionine, homocysteine, and S-benzylhomocysteine, to their corresponding keto acids and methylamine.

Ratner, Nocito, and Green (3) have reported that glycine oxidase, which has been found in the liver or kidney of animals, catalyzes the aerobic oxidation of sarcosine to glyoxylic acid and methylamine.

Although these oxidations of N-monomethylamino acids are all oxidative demethylaminations, Handler, Bernheim, and Klein (4) have presented further evidence of the direct demethylation of sarcosine. Sarcosine oxidase which was found in rat, rabbit, and guinea pig livers oxidatively demethylates sarcosine to glycine and formaldehyde.

During the course of our investigations of the metabolism of N-methyl derivatives of amino acids, it has been demonstrated that the broken cell preparations of the kidney and liver of the rabbit, guinea pig, pigeon, and hen oxidize the N-methyl-L-tryptophan, obtained from *Abrus precatorius*, to L-tryptophan and formaldehyde by the direct demethylation which was due to the presence of demethylase (5). Furthermore it has been shown that demethylase is a flavoprotein (6) which can be resolved into a protein and adenine-flavin-dinucleotide. Recently, in the course of our studies on the specificity of demethylase, it was evident that demethylase can also demethylate other N-monomethyl-L-amino acids, which have been tested, to their corresponding amino acids and formaldehyde and cannot oxidize N-monomethyl-D-amino acids and N-dimethylamino acids.

Some of the results are given in Table I.

\* The former name was Tai-sei To or Tatuo Yosida.



Both sarcosine oxidase and demethylase are completely analogous in their action on  $\alpha$ -methylamino acids, but they are unquestionably different enzymes.

TABLE I  
*Specificity of Demethylase\**

Substrate	Oxygen† absorbed	Formaldehyde‡ formed
	c.mm.	mg.
N-Methyl-L-tryptophan . . . . .	81	0.187
N-Methyl-D-tryptophan. . . . .	0	0
D-Tryptophan . . . . .	0	0
O,N-Dimethyl-DL-tyrosine. . . . .	65	0.162
O,N-Dimethyl-D-tyrosine. . . . .	0	0
N-Methyl-DL-tyrosine . . . . .	58	0.124
L-Tyrosine. . . . .	0	0
N-Methylvanillyl-DL-alanine. . . . .	48	0.110
N-Methyldihydroxyphenyl-DL-alanine . . . . .	53	0.121
N-Methyl-DL-phenylalanine. . . . .	52	0.123
N-Methyl-D-phenylalanine . . . . .	0	0
DL-Phenylalanine. . . . .	0	0
N $\alpha$ -Methyl-DL-histidine . . . . .	60	0.139
L-Histidine . . . . .	0	0
N-Methyl-L-leucine. . . . .	24	0.058
L-Leucine. . . . .	0	0
N-Methyl-DL-alanine . . . . .	12	0.025
Sarcosine. . . . .	0	0
N-Dimethyl-p-methoxy-DL-phenylalanine. . . . .	0	0
N-Dimethyl-DL-phenylalanine. . . . .	0	0

\* The enzyme was prepared as follows. The rabbit kidney was desiccated with acetone. 2 gm. of the kidney powder were suspended in 40 cc. of 0.9 per cent NaCl and the mixture was filtered. The filtrate was treated with 0.8 cc. of 10 per cent basic lead acetate. The supernatant fluid was then precipitated twice with the same volume of saturated ammonium sulfate of pH 6.8. The precipitate was resuspended in 10 cc. of 0.9 per cent NaCl and the supernatant fluid was used as the enzyme.

† Oxygen uptakes were measured manometrically in the Warburg apparatus. The manometer cup contained 0.5 cc. of enzyme, 1 cc. of 0.02 M N-methylamino acid, and 2 cc. of M/15 phosphate buffer of pH 7.1 (38°, 60 minutes).

‡ Formaldehyde was estimated colorimetrically by a modification of the phenylhydrazine-ferric chloride test.

#### SUMMARY

Production of formaldehyde by oxidative demethylation of N-mono-methyl-L-amino acids by demethylase appears to be a general reaction. N-Dimethyl-L-amino acids and N-methylated D-amino acids yield no formaldehyde under the influence of demethylase.

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# ISOLATION OF THE UNIDENTIFIED GROWTH FACTOR (VITAMIN B<sub>12</sub>) IN DISTILLERS' DRIED SOLUBLES\*

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Recent investigations have shown that distillers' dried solubles contain a factor or factors which are necessary for the growth of chicks (1) and rats (2). This factor is distinct from vitamin A, vitamin D, thiamine, riboflavin, inositol, nicotinic acid, pantothenic acid, *p*-aminobenzoic acid, choline, pyridoxine, biotin, folic acid, or 2-methylnaphthoquinone.

A procedure has been developed for the isolation of this unidentified growth factor in a highly concentrated condition. This method is based upon physical and chemical characteristics of the active principles which were discovered during the fractionation studies (2).

## EXPERIMENTAL

A method was developed for the isolation of the unidentified growth factor in distillers' dried solubles, which involved the following essential procedures: extraction of distillers' dried solubles with acidified water, removal of proteins, differential chromatographic adsorption of impurities, precipitation, and the separation of the active principle with an immiscible solvent. The activity of the various fractions was determined by the rat growth method previously described (2).

Distillers' dried solubles were extracted with 3 liters of 0.1 N HCl per kilo of solubles by autoclaving for 30 minutes at 120°. This preparation was cooled, adjusted to pH 6.5, and filtered. The residue was reextracted twice, after which it was washed with distilled water by stirring and again filtered. The combined filtrates were concentrated under reduced pressure to the equivalent of approximately 200 ml. per kilo of original material and adjusted to pH 3.5.

To precipitate protein and other inert material, this concentrate was poured into 3 volumes of ethanol, stirred for several minutes, allowed to stand overnight, and filtered. Under these conditions, factor S (3) should

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have been removed. The filtrate was adjusted to pH 7, stirred, allowed to stand overnight, and again filtered. This procedure is reported to remove factor R (3).

The filtrate was slightly acidified with hydrochloric acid, concentrated under reduced pressure to about 150 ml. per kilo of original material, and adjusted to pH 6.5. This solution was filtered through a column (4 cm.  $\times$  10 cm.) of fullers' earth<sup>1</sup> diluted with Celite (1:1) which removed some impurities without adsorbing the active principle (2).

The filtrate was concentrated under diminished pressure to about 100 ml. per kilo of the original solubles. After adjusting the solution to a hydrochloric acid content of 3.5 per cent, sufficient solution of phosphotungstic acid (30 gm. per 100 ml. of 3.5 per cent HCl) was added for complete precipitation. The contents of the flask were heated to dissolve most of the precipitate, cooled, and placed in the refrigerator for 48 hours. The cold solution was filtered through a cold Büchner funnel with a hardened filter paper (Whatman No. 50). The precipitate was washed several times with a cold solution containing 2.5 per cent phosphotungstic acid, and 3.5 per cent hydrochloric acid. The filtrate was found to be biologically inactive, and was discarded.

The precipitate was dissolved in a slightly alkaline solution of sodium hydroxide, and the phosphotungstic acid precipitated with barium chloride. The barium phosphotungstate was removed by filtration, and washed with hot water.

After concentration, the aqueous solution was extracted repeatedly with chloroform in a separatory funnel. The combined chloroform extracts were concentrated to dryness under reduced pressure. A yellow-orange non-crystalline residue remained. This material was found to be readily soluble in water, acetone, ethanol, ethyl ether, chloroform, and benzene. When this material was dissolved in water at a concentration of 100  $\gamma$  per ml., it appeared practically colorless and showed fluorescence under an ultraviolet lamp.

To determine its biological activity, this material was added to the basal ration at approximately 250  $\gamma$  per 100 gm. of ration and tested with rats as previously described. A good growth response was obtained (Table I).

Since the aqueous solution was almost colorless, its spectral absorption characteristics were studied. A Beckman spectrophotometer, equipped with a hydrogen arc and quartz cells, was used at wave-lengths between 2400 and 4000 Å. To determine whether the absorption curve bore any relation to the active component from distillers' solubles, concentrates were prepared from other active materials and their spectral characteristics were compared (Fig. 1). Concentrates prepared from rice polishings concentrate

<sup>1</sup> City Chemical Corporation, New York.

TABLE I  
*Biological Tests for Presence of Growth Factor*

Concentrates added to basal ration*	Average gain per wk.† (8 rats per lot)
	gm.
None.....	17.2
Distillers' solubles (Seagram).....	29.8
Liver extract (Lilly).....	27.8
Rice polishings (Labco).....	28.0

\* In amounts equivalent to approximately 10 per cent of the original material.

† Least significant difference, 5 per cent level 2.5 gm., 1 per cent level 3.4 gm.

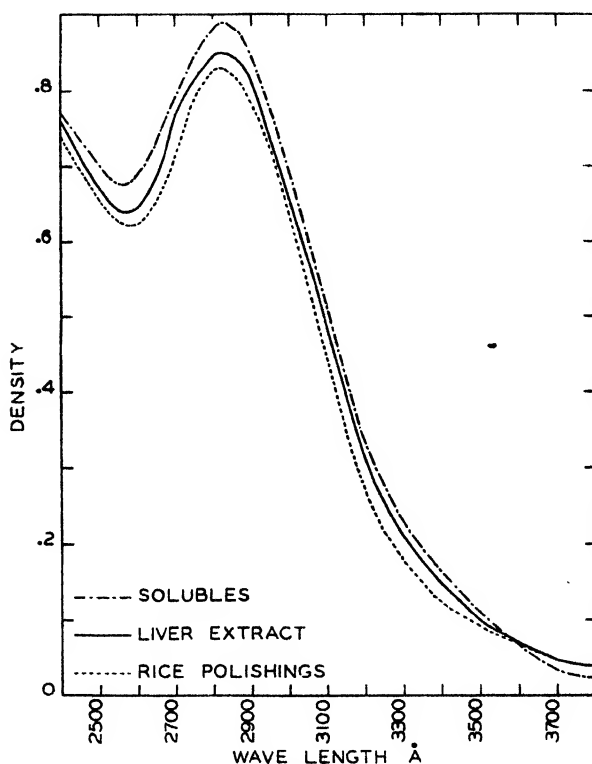


FIG. 1. A comparison of the absorption spectra of the isolated concentrates from distillers' dried solubles, rice polishings concentrate (Labco), and liver extract (Lilly).

(Labco) and liver extract (Lilly) were diluted so that at the maximum of 2820 Å they gave densities slightly less than that obtained with the preparation from the distillers' solubles. The biological activities of these con-

concentrates (Table I) established a parallelism between the characteristic curves and activity.

After it was found that maximum growth response was produced with 250  $\gamma$  of the isolated material from solubles per 100 gm. of the ration, a biological assay was performed to determine the minimum quantity necessary for a definite growth response and the amount required for a maximum biological response.

The biological assay was performed with 50 rats, equally divided according to sex. Instead of incorporating the factor into the ration, it was fed

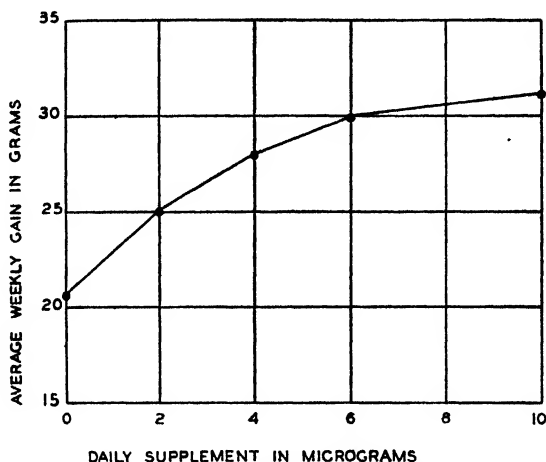


FIG. 2. The growth response of rats fed the basal ration and daily supplements of the unidentified factor.

as a daily supplement. In a preliminary assay, it was found that levels of 10, 20, 30, and 40  $\gamma$  per day were equally effective in stimulating growth. This indicated that the effective dose was below 10  $\gamma$  per day. Therefore levels of 0, 2, 4, 6, and 10  $\gamma$  per day were fed. Ten rats were fed at each level. The growth response curve is given in Fig. 2. The maximum biological effect was obtained with 10  $\gamma$  of the factor per day. Definite growth stimulation was obtained with 2  $\gamma$  per day.

Since the characteristic absorption spectra of the concentrates from the three different sources were practically identical, exhibiting a maximum at 2820 A, and inasmuch as they were biologically active, it is apparent that the isolation of the unidentified growth factor in a high state of purity has been accomplished.

#### DISCUSSION

Although previous experiments (1, 2) gave strong evidence for the existence of an unidentified growth factor in distillers' dried solubles which was

distinct from the known vitamins and postulated factors, these experiments offer proof that the observed growth responses were due to a definite substance which has been isolated in a state of relatively high purity. This is substantiated by its characteristic absorption spectrum and by its high biological activity. Since the potency of this product is comparable to that of a number of vitamins, it appears logical to class this factor with the other vitamins of the B complex. It is tentatively called vitamin B<sub>13</sub>.

A number of properties are now known. It is stable to heat, acid, and alkali. It is soluble in water, acetone, chloroform, ethanol, ethyl ether, and benzene. It is precipitated by phosphotungstic acid and lead acetate. It is not adsorbed on fullers' earth or Darco but is adsorbed from acid solution on Florisil, Lloyd's reagent, norit, and Decalco. Its absorption spectrum exhibits a maximum at 2820 Å.

The solubility of this factor in chloroform as well as ether adds further evidence that it is not the cow manure factor (4). It is possible that it may be similar to one of the fractions obtained from liver extract by Barton-Wright *et al.* (5). They obtained a fraction by the extraction of an aqueous solution with chloroform, which stimulated growth of *Lactobacillus helveticus* and *Streptococcus lactis*. No tests were reported with animals.

#### SUMMARY

A new growth factor for rats, tentatively called vitamin B<sub>13</sub>, has been obtained from distillers' dried solubles, rice polishings concentrate, and liver extract in a non-crystalline but highly purified state. 2 γ of this substance give definite growth stimulation and 10 γ per day give the maximum effect.

Spectral absorption curves showing a maximum at 2820 Å were obtained with concentrates of high potency from each of the three materials, distillers' dried solubles (Seagram), rice polishings concentrate (Labco), and liver extract (Lilly).

The procedure for isolation involves extraction with 0.1 N hydrochloric acid, precipitation of protein and other inert material with ethanol, chromatographic adsorption of impurities on fullers' earth, precipitation of the active factor with phosphotungstic acid, and separation by chloroform extraction.

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# THE COMBINATION OF ORGANIC ANIONS WITH SERUM ALBUMIN

## VI. QUANTITATIVE STUDIES BY EQUILIBRIUM DIALYSIS

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(Received for publication, February 2, 1948)

Electrophoretic studies have shown that a combination occurs between serum albumin and lower fatty acids (1), synthetic detergents (2-4), and certain buffer anions (5-10). In addition, serum albumin has been shown to combine with phenol red (11, 12), sulfonamides (13, 14), penicillin (15), and calcium ion (16). Quantitative studies on the serum albumin combination with lower fatty acids have been attempted earlier in this laboratory by ultrafiltration (17).

The combination of methyl orange and an azo dye with serum albumin has been investigated by the dialysis-equilibrium technique by Klotz *et al.* (18).

The results of further dialysis-equilibrium studies on the nature and extent of the combination of serum albumin with some aromatic carboxylates and nitrophenolates are reported in the present paper.

### EXPERIMENTAL

*Protein Solutions*—Crystalline bovine serum albumin was used in all cases with one exception: an amorphous albumin was used in the experiments with the phenyl butyrate anion. These albumin preparations were obtained from the Armour Laboratories through the courtesy of Dr. J. D. Porsche.

The stock protein solutions were prepared by either of the two following methods: (a) a quantity of albumin was dissolved in water with the aid of a vacuum, as described by Duggan and Luck (19), to give approximately a 20 per cent solution by weight; (b) a sufficient quantity of albumin was dissolved in water to give approximately a 2 per cent solution by weight.

The exact concentration was obtained by a dry weight determination on a sample of the solution dried at 110° for 24 hours.

Solutions of buffered protein of desired concentration (usually about 0.2 per cent) were prepared by diluting portions of the concentrated stock solution with phosphate buffer. The ionic strength of the buffer was 0.2 at pH 7.6 and 0.22 at pH 8.2.

The sodium salts of the compounds reported in this paper were prepared

from the corresponding acids by neutralization with sodium hydroxide. Solutions of these salts, 0.3 or 0.5 M, were diluted with phosphate buffer to concentrations ranging from  $5 \times 10^{-3}$  M to  $100 \times 10^{-5}$  M.

*Dialysis*—Commercial sausage casing was used in the preparation of cellophane bags for dialysis. In some cases, the bags were boiled three times for  $\frac{1}{2}$  hour and rinsed with distilled water after each boiling period.

TABLE I

*Dialysis Equilibrium between Bovine Serum Albumin and Phenyl Butyrate Anion*

Tube No.	Molarity of anion outside of bag	Bound A <sup>-</sup>	$\frac{\text{Moles bound A}^-}{\text{Moles Pr}^*} = r$	$\frac{1}{r}$	Free A <sup>-</sup>	$\frac{1}{A^-}$
	$\times 10^3$	moles $\times 10^3$			moles $\times 10^3$	$\times 10^{-3}$
1	14.5	25.5	1.76	0.57	21.75	0.46
1a†	16.2					
2	17.0	30	2.07	0.483	25.5	0.392
2a	19.0					
3	27.8	54	3.72	0.269	41.7	0.24
3a	31.4					
4	34.3	55.5	3.82	0.262	51.4	0.194
4a	38.0					
5	41.2	66	4.55	0.22	61.8	0.162
5a	45.6					
6	48	75	5.17	0.194	72	0.139
6a	53					
7	70	90	6.20	0.161	105	0.095
7a	76					

Volume of anion placed outside of bag, 10 ml.; volume of albumin placed inside of bag, 5 ml.

\* The number of moles of protein was calculated on the assumption that the molecular weight of serum albumin is 70,000. 5 ml. are equivalent to  $1.45 \times 10^{-7}$  mole.

† The control series, designated "a," contained 5 ml. of buffer only inside of the bag.

This procedure was found necessary when the spectrophotometric analysis of the anion involved the ultraviolet region.

Cellophane bags containing 5 ml. of 0.2 per cent buffered protein solution were immersed in 10 ml. of each anion solution contained in a suitable bottle. Controls containing buffer only inside the bag were prepared in the same manner for each concentration of anion. The bottles were placed in a cold room at 1° for 3 days (an interval sufficient to insure equilibrium). The bags were then removed and the external solutions were analyzed spectrophotometrically for the anion. The results of a typical dialysis experiment are shown in Table I.

## RESULTS AND DISCUSSION

The treatment of the data obtained was similar to that applied to methyl orange and an azo dye by Klotz *et al.* (18).

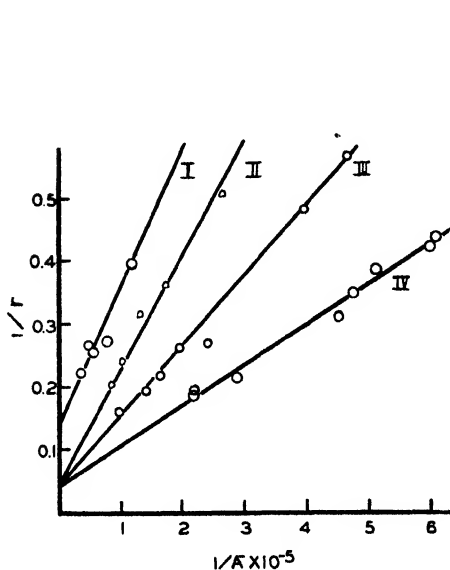


FIG. 1

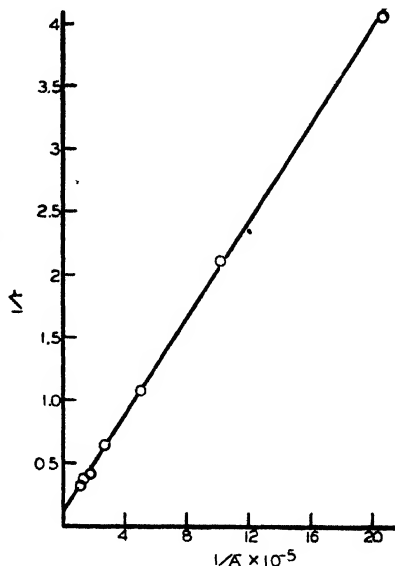
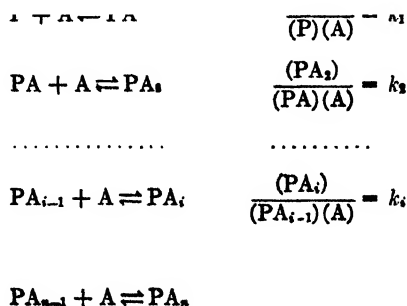


FIG. 2

FIG. 1. The binding of sodium phenoxyacetate (Curve I), sodium phenyl acetate (Curve II), sodium phenyl butyrate (Curve III), and sodium cinnamate (Curve IV) with bovine serum albumin at pH 7.6 and ionic strength 0.2.

FIG. 2. Bovine serum albumin-sodium hippurate at pH 7.6 and ionic strength 0.2.

The successive protein-anion equilibria may be represented by a series of equations shown below.



P represents a molecule of free protein and A a molecule of organic anion;  $n$  represents the maximum possible number of bound anions per protein

molecule. The classical equilibrium constants are given by the relations shown on the right (above).

It may also be shown that the ratio  $r$ , moles of bound anion to the total moles of protein, is given by the equation

$$r = \frac{k_1(\Lambda) + 2k_1k_2(\Lambda)^2 \cdots + n(k_1k_2 \cdots k_n)(\Lambda)^n}{1 + k_1(\Lambda) + k_1k_2(\Lambda)^2 \cdots + (k_1k_2 \cdots k_n)(\Lambda)^n} \quad (1)$$

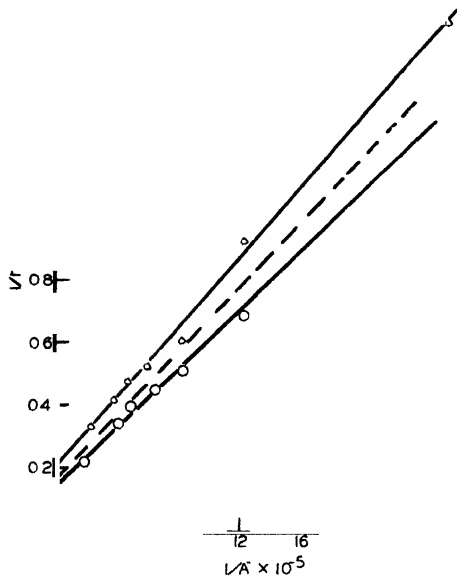


FIG. 3

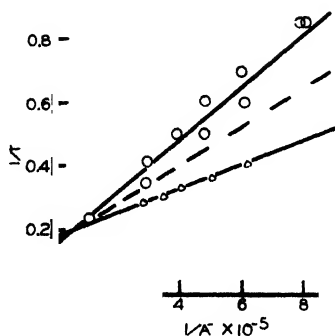


FIG. 4

FIG. 3. Bovine serum albumin-sodium *o*-nitrophenolate at pH 7.6 and ionic strength 0.2. The broken line represents the average of the experimental curves.

FIG. 4. Bovine serum albumin-sodium *o*-nitrophenolate at pH 8.2 and ionic strength 0.22. The average curve is shown by the dotted line.

This involved expression may be simplified in cases of negligible electrostatic repulsion between bound and free anion. For this situation

$$\frac{n - (i - 1)}{i} \frac{1}{K} \quad (2)$$

$$k_1 = K \quad (3)$$

$K$  is a constant which depends on the nature of the anion as well as on the character of the protein, and hence must be determined experimentally. It can be shown that when statistical effects are predominant and the

equilibrium constants are given by equation (2) the relation for  $r$  in equation (1) may be reduced to a linear form.

$$\frac{1}{r} = \frac{K}{n} \frac{1}{(A^-)} + \frac{1}{n} \quad (4)$$

Thus, if a straight line results when  $1/r$  is plotted against  $1/(A^-)$ , statistical factors predominate. All cases reported in this paper followed this

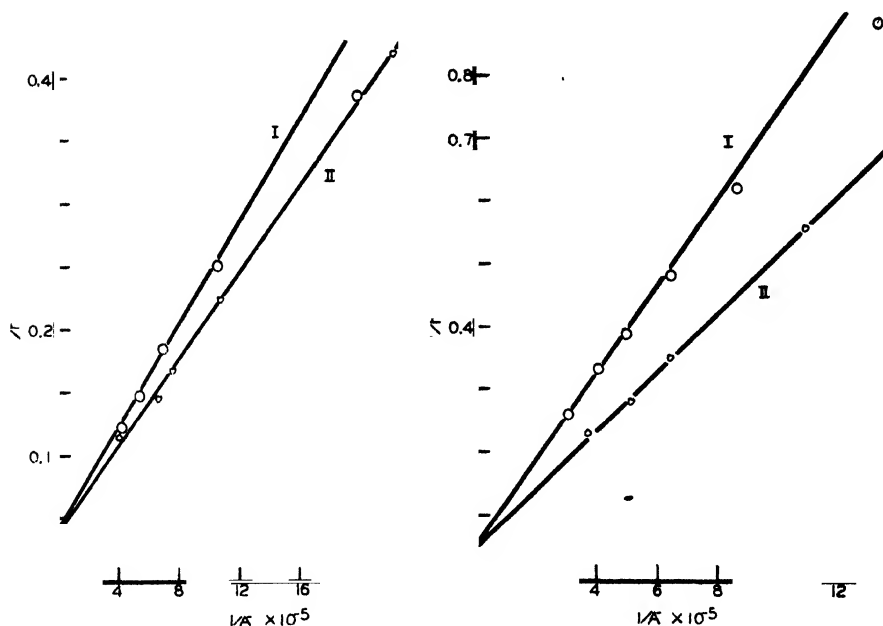


FIG. 5

FIG. 6

FIG. 5. Bovine serum albumin-sodium *p*-nitrophenolate (Curve I) and bovine serum albumin-sodium *m*-nitrophenolate (Curve II) at pH 7.6 and ionic strength 0.2.

FIG. 6. Bovine serum albumin-sodium *p*-nitrophenolate (Curve I) and bovine serum albumin-sodium *m*-nitrophenolate (Curve II) at pH 8.2 and ionic strength 0.22.

straight line relation, as is shown in Figs. 1 to 8. The curves are drawn to give emphasis to the points of greatest accuracy (high anion concentration or low  $1/(A^-)$  values).

The intercept when  $1/(A^-)$  is zero gives the value for  $n$ . The values  $k_1$ ,  $K$ , and  $\Delta F_1$  were calculated from the foregoing equations for the anions shown in Table II. The results indicate that the length as well as the type of side chain affects the extent and the strength of the binding. An increase in the number of carbon atoms in the chain gave an increase in  $\Delta F_1$ . An increase in the chain length by addition of an oxygen atom or a

peptide group caused an increase in the  $\Delta F_1$  value but caused a decrease in the extent of binding as shown by the values for  $n$ .

It is interesting to note that results obtained in the study of stabilization against urea denaturation of albumin showed that phenyl butyrate and cinnamate were good stabilizers and that the others listed in Table II were comparatively poor (19). The introduction of polar groups in the side chain of the aromatic carboxylates decreased the stabilizing effect of the compound. This is in accord with the decreased value for  $n$  obtained in the dialysis-equilibrium studies. The increase in the energy of the binding of cinnamate, phenyl butyrate, phenoxyacetate, and hippurate

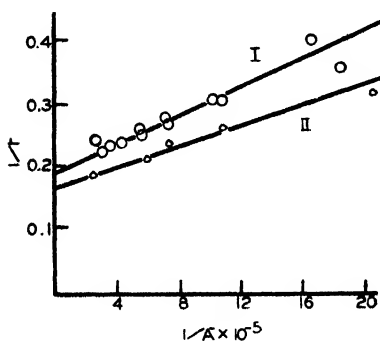


FIG. 7

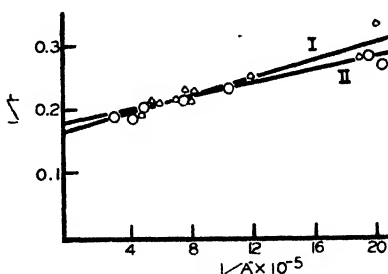


FIG. 8

FIG. 7. Bovine serum albumin-sodium 2,4-dinitrophenolate at pH 8.2 and ionic strength 0.22 (Curve I). Curve II represents the same system at pH 7.6 and ionic strength 0.2.

FIG. 8. Bovine serum albumin-sodium picrate at pH 8.2 and ionic strength 0.22 (Curve I). Curve II represents the same system at pH 7.6 and ionic strength 0.2.

over the binding energy of phenyl acetate cannot be explained simply by the contribution due to van der Waals' forces.

In recent papers (20-22), the ability to stabilize albumin was attributed to the presence of a non-polar radical in the molecule. It was later found that picrate, trichloroacetate, and dichlorophenolate are good stabilizers against urea denaturation (19). These findings aroused interest in the application of dialysis-equilibrium measurements to some nitrophenols, including picrate.

The values for the various constants, calculated as above, are presented in Table III. Compounds containing a nitro group in the ortho position exhibit a value for  $n$  of 6 as compared to 22 to 25 for the other compounds. The low value for the ortho-substituted compounds cannot be explained without knowledge of the protein groups involved in the binding and the arrangement of these active groups in the molecule.

The energy of the binding of the first picrate and of the first 2,4-dinitrophenolate anion is 8900 calories per mole at pH 7.6. The energy at pH 7.6 for the mononitro compounds ranges from 7150 to 7850 calories per mole. The energy increases with increase in pH in the cases of *m*-nitro- and *p*-nitrophenolate, but it decreases with increase in pH in compounds which are almost completely in the anion form.

Since, as previous evidence indicates, organic anions are bound to the

TABLE II  
*Binding of Some Aromatic Carboxylates with Bovine Serum Albumin*

Anion	<i>n</i>	<i>K</i>	<i>h</i> <sub>1</sub>	-Δ <i>F</i> <sub>1</sub>
		× 10 <sup>4</sup>	mole × 10 <sup>-4</sup>	calories per mole
Phenyl butyrate	24	2.64	8.92	7450
Cinnamate	25	1.6	15.7	7750
Hippurate	9	1.51	5.66	7220
Phenyl acetate	25	23.0	1.09	6300
Phenoxyacetate	7	1.35	5.18	7150

TABLE III  
*Binding of Nitrophenolates with Bovine Serum Albumin*

Anion	pH	<i>n</i>	<i>K</i>	<i>h</i> <sub>1</sub>	-Δ <i>F</i> <sub>1</sub>	A <sup>-</sup> form
			× 10 <sup>4</sup>	× 10 <sup>-4</sup>	calories per mole	per cent
Nitrophenolate	7.6	6	0.338	1.82	7850	75
	8.2	6	0.407	1.51	7740	91
Nitrophenolate	7.6	24	4.05	0.603	7250	16
	8.2	22	1.11	2.0	7900	43
Nitrophenolate	7.6	25	5.12	0.49	7150	72.5
	8.2	25	1.69	1.48	7750	91
1-Dinitrophenolate	7.6	6	0.047	12.5	8900	100
	8.2	6	0.068	8.0	8650	100
crate	7.6	6	0.048	12.6	8900	100
	8.2	6	0.052	11.6	8850	100

positive groups of the protein, and since an increase in pH increases the negative charge on the protein, a repulsion between protein and anion would result when van der Waals' forces do not predominate.

In conclusion, the results indicate that in the case of the aromatic carboxylic acids an increase of the length of the side chain by the addition of carbon atoms increases the energy of the binding. An increase in chain length by the addition of an oxygen or a peptide group increases the energy but decreases the extent of the binding. In the case of the nitrophenolates, a nitro group in the ortho position decreases the extent of the binding as



compared to a nitro group in the para and meta positions. Addition of a nitro group to *o*-nitrophenolate increased the affinity of the anion. A second additional nitro group to the *o*-nitrophenolate had no effect at pH 7.6 but increased the strength of the bond at pH 8.2. Finally, an increase of pH decreases the energy of the binding when 100 per cent of the compound is in the anion form and this decrease is due to the repulsion caused by an increase in the negative charge on the protein.

#### SUMMARY

The law of mass action has been applied to dialysis-equilibrium data obtained from the systems, bovine serum albumin-aromatic carboxylic acids and bovine serum albumin-nitrophenolates. In all the cases studied, it was found that statistical factors predominate; *i.e.*, electrostatic repulsion between bound and free anions was negligible.

The maximum number of molecules of each anion studied that can combine with 1 molecule of bovine serum albumin has been determined. The energy of the binding of the first anion was determined for each compound. The value for the constant of equilibrium between the first anion and the protein was also calculated.

The effect of the type and length of side chain in the aromatic carboxylates on the extent and energy of binding to the albumin has been discussed.

A discussion of the effect of the position of the nitro group in nitro phenolates on the extent and energy of binding is reported.

We are indebted to the Armour Laboratories for the albumin preparations and to Dr. Frank W. Allen of the University of California for the *p*- and *m*-nitrophenolates and for the 2,4-dinitrophenolate used in these studies.

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# THE STATE AND DISTRIBUTION OF STEROID HORMONES IN BIOLOGIC SYSTEMS

## III. SOLUBILITIES OF TESTOSTERONE, PROGESTERONE, AND $\alpha$ -ESTRADIOL IN AQUEOUS SALT AND PROTEIN SOLUTION AND IN SERUM\*

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Except for such complications as change in activity coefficient, change in state of aggregation, selective membrane permeability, or chemical reaction, the property primarily governing the distribution of a substance in a polyphase system, such as occurs in the living organism, is solubility. In spite of the recent extensive progress in the organic chemistry of the steroids, meager information is available regarding the physical properties of these compounds. The present report is concerned with the solubilities of testosterone, progesterone, and  $\alpha$ -estradiol in aqueous solutions of varying ionic strength, in aqueous protein solutions, and in serum. This information is required as a basis for speculation as to and investigation of the physiochemical status and fate of these substances in the organism.

### EXPERIMENTAL

*Equilibration*—Crystalline Schering testosterone (m.p. 153.7–154.2°, corrected) and progesterone (m.p. 127.5–128.5°, corrected), which suffered no loss in weight on desiccation over  $P_2O_5$ , were used in all experiments. The  $\gamma$ -globulin was prepared in the laboratories of Armour and Company and was electrophoretically homogeneous at pH 7.0. The albumin was the crystalline bovine Armour product. Saturation was approached from both undersaturation and supersaturation at two temperature ranges, viz., 25.0° and 37.5°. Equilibration at 37.5° was performed during the day in a thermostatically controlled cabinet equipped with a mechanical shaker and at night in a water bath. Equilibration at 25.0° was performed in a water bath; agitation of the solvent depended upon the rotation of the suspended container with the currents set up by the stirrer. In the progesterone experiments, a thermostatically controlled cabinet with mechanical shaker was also used at 25.0°. Filtration was performed in thermostatically

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For the first two papers of this series see (10, 11).

controlled cabinets. Effect of concentration of solute, which is a criterion for purity of the solute, was studied over a 4-fold range. Effect of change of solvent, which is likewise a criterion of purity, was studied for three or four changes. The solvents studied were water, 1.0, 1.97, or 3.89 per cent aqueous NaCl, 0.1 per cent aqueous  $\text{NaHCO}_3$ , 1.97 or 3.87 per cent aqueous  $\text{Na}_2\text{SO}_4$ , 1 or 3 per cent crystalline bovine albumin solution at  $\mu = 0.155$ , 1 or 3 per cent bovine  $\gamma$ -globulin at  $\mu = 0.155$ , and rabbit serum. The influence of pH over the range 5.0 to 8.5 was studied in certain instances. The details are given in the respective tables.

In working with protein solutions aseptic technique was used. Non-protein nitrogen determinations were performed upon protein-free filtrates as a gage of possible bacterial contamination and action.

*Method of Determination*.—The color reaction of Zimmermann (1), depending upon the reaction of certain ketones with *m*-dinitrobenzene in alkaline alcohol solution, has been utilized by Holtorff and Koch (2) for the determination of 17-ketosteroids and related compounds and was adapted for the determination of testosterone and progesterone in the present studies. By means of the Lumetron colorimeter with voltage stabilizer for photocolormetry, the yellow-green filter (wave-length 530  $\text{m}\mu$ ) gave values for testosterone and progesterone in conformity with Beer's law over the concentration range of 0.05 to 0.2 mg. The error of the instrument in the maximum range of sensitivity was about 1 per cent.

The concentration of testosterone from water or aqueous salt solution in amounts required for the colorimetric determination was performed by evaporation of the aqueous or salt solution at 99°; the residue was extracted with ethanol to separate salts and the filtrate subjected to evaporation at the same temperature. In the case of progesterone this procedure was not feasible, as a variable recovery ranging from 60 to 93 per cent (seven experiments) was encountered on evaporation from NaCl solution at 99°, and therefore the aqueous phase was concentrated at room temperature, *in vacuo*, over calcium chloride.

It was planned to study the effect of the  $\text{HPO}_4^{=}$  ion on the salting-out phenomenon, but this study had to be abandoned because of the analytical difficulties encountered in the determination of testosterone in the presence of phosphate; the phosphate cannot be dehydrated at temperatures at which testosterone is stable, and too large a quantity enters into the alcoholic extract.

The justification of the analytical procedures used is based upon the following experimental data. The recovery of 0.2 mg. of testosterone subjected to evaporation of 5 or 10 cc. of water or absolute ethanol in a bath at 99° was 98 to 102 per cent (twelve determinations) when compared with a 0.2 mg. standard added to the residue obtained by evaporating water or

alcohol. The recovery after subjecting the testosterone to evaporation from 3.9 per cent NaCl solutions with subsequent alcohol extraction and evaporation was  $96 \pm 1$  per cent in nine of twelve experiments compared with standards added after the aqueous evaporation, alcohol extraction, and alcohol evaporation had been performed to serve as a blank. Similar values were obtained from 4 per cent sodium sulfate solution. The recovery of progesterone, when evaporation of the aqueous phase was performed *in vacuo*, was  $97 \pm 0.6$  per cent (eight determinations). The recovery from 4 per cent  $\text{Na}_2\text{SO}_4$  solution was 99 per cent (two determinations). A stock standard in absolute ethanol containing 2 mg. of progesterone per cc. lost no activity in 8 months when compared with a fresh standard. The possibility of loss of dissolved solute by adsorption on filter paper was eliminated by comparison of filtration and centrifugation at  $25^\circ$ .

*Reagents*—Redistillation of commercial absolute and 95 per cent ethanols reduced the blank appreciably. The water was distilled with a Barnsted still and then double distilled from glass. A solution of Eastman *m*-dinitrobenzene, 2 per cent by volume in 95 per cent ethanol, recrystallized by the method of Callow (3), was used.  $5 \pm 0.04$  N aqueous KOH was prepared from a saturated solution of Baker's c.p. KOH, decanted from the carbonate precipitate. Both Munktell No. 00 and Whatman No. 2 filter papers, which were used for filtration, gave a measurable blank (one-tenth to one-fifth of the total blank). The Munktell blank was due to a water-soluble substance; the Whatman blank to an alcohol-soluble material.

*Procedure for Testosterone for Aqueous or Aqueous Salt Solution*—10.0 cc. aliquots obtained by filtration through filter paper in a cabinet maintained at the equilibration temperature plus 1.0 cc. of absolute ethanol are evaporated to dryness at the temperature of the water bath in a narrow mouthed bottle. After cooling, 10.0 cc. of absolute ethanol are added to each, the precipitate is broken up with a nichrome rod, and the contents are allowed to stand 1 hour and then filtered. A 5.0 cc. aliquot of the filtrate is evaporated to dryness in the photometer tube on the water bath. The residue is dissolved in 0.5 cc. of absolute ethanol. 0.5 cc. of dinitrobenzene reagent and 0.5 cc. of KOH reagent are added. The whole is mixed and set aside in the dark for 45 minutes, when 7 cc. of 95 per cent ethanol and 1.5 cc. of water are added. The mixture is allowed to stand 3 minutes and then read immediately with standards which are processed in exactly the same manner as the unknowns. The effect of the small amount of salt which dissolves in the absolute ethanol and the effect of salt upon the alcohol extraction are controlled by processing the standards for the solubility in salt solutions by adding 1.0 cc. of the ethanol solution of the standards to 10 cc. of 3.89 aqueous NaCl solution. In determinations

from solution at pH 9.1 the aliquot was titrated to pH 5.0 with 0.2 N HCl before processing.

*Procedure for Aqueous and Aqueous Salt Solution of Progesterone*—20.0 cc. aliquots plus 1 cc. of absolute ethanol are evaporated to dryness in 180 cc. narrow mouth bottles *in vacuo* at room temperature over calcium chloride. The standards in a volume of 1 cc. of absolute ethanol are added to 20 cc. portions of a blank solution and processed simultaneously with the unknowns. After evaporation to dryness the process is the same as that for testosterone. The concentrations of the standards in the determination are 0.2 and 0.05 mg.

*Calculation*—The optical densities of the high and low standards (0.2 and 0.05 mg. respectively) are plotted against their concentrations on ordinary graph paper. The optical densities of the unknowns are referred to a line joining the coordinate values for the standards. Alternately if  $A_1$  and  $A_2$  are the optical densities of the high and low standards respectively (0.2 and 0.05 mg.) and  $X$  is the intercept at 0 concentration,

$$A_1 - X = 4(A_2 - X)$$

$$X = \frac{4A_2 - A_1}{3}$$

If the optical density of the unknown is  $B$ , then

$$\frac{B - X}{A_1 - X} \times 0.2 = \text{mg. steroid in unknown sample}$$

*Recovery of Testosterone from Protein Solution*—When 5 cc. of 3 per cent crystalline bovine albumin in 0.8 per cent NaCl plus 0.4 mg. of testosterone were evaporated to dryness on the water bath or *in vacuo* at room temperature, extraction of the residue with 10 cc. of absolute ethanol for 2 hours failed to recover any testosterone. Extraction with 10 cc. of chloroform also proved unsatisfactory. When an albumin solution containing testosterone was added to absolute ethanol so that the alcohol content was 80 per cent by volume, the testosterone was quantitatively recovered from the alcoholic filtrate (five experiments) when compared with a standard in which the testosterone was added to the alcoholic filtrate after removal of protein. In the analytical determination, the alcoholic filtrate is evaporated to dryness on the water bath. The process for recovering testosterone from rabbit serum was similar to that used for the albumin solution. The concentration of the ethanol solution was raised to 90 per cent by volume and the mixture was permitted to stand overnight before filtration. Recovery in four experiments was 104 per cent when comparison was made with a standard in which the testosterone was added after filtration of the al-

coholic solution. The extractives from bovine albumin and rabbit serum were tested for their effect upon the color development of testosterone at 0.05, 0.1, and 0.2 mg. concentration. The deviation from Beer's law was 4 per cent at the highest concentration for each substance (each experiment in triplicate). Since standards prepared with alcoholic filtrates of rabbit serum or albumin were used in all solubility determinations, this effect was controlled.

The process for assaying progesterone in albumin solution or in rabbit serum was the same as that used for testosterone. In three experiments the recovery from albumin solution was 101 per cent when comparison was made with a standard in which progesterone was added to the alcoholic filtrates. In four experiments the recovery from serum was 101 per cent when comparison was made with a standard in which the progesterone was added to the alcoholic filtrate after precipitation of protein. The influence of the alcohol-soluble constituents of albumin or serum upon the color development was exactly comparable to that found for testosterone. Recovery of both testosterone and progesterone, added to globulin before processing for analysis, was 100 to 102 per cent of the value found after adding these hormones to the respective protein filtrates. The accuracy of the analytical procedure was of the same order as that found for albumin.

Attempts at ultrafiltration with collodion membranes were unsuccessful, since both hormones as well as estradiol were adsorbed by the membrane. The adsorption of testosterone and progesterone was virtually complete from a saturated aqueous isotonic solution. 90 to 99 per cent of the hormones could be recovered by extraction of the membrane with 70 per cent ethanol in the case of testosterone and with 100 per cent ethanol (but not 70 per cent) in the case of progesterone. Testosterone was not adsorbed by lamb's cecum (Julius Schmidt Fourex) and was quantitatively recovered in the ultrafiltrate.

*Accuracy of Procedure*—In twenty-eight pairs of determinations of testosterone from water or aqueous sodium chloride solution in which each sample was processed independently with its own set of standards, the mean deviation from the mean of the pair was 3.1 per cent, 86 per cent of the values deviating not more than 5 per cent from the mean. The accuracy of the determination was greatly increased in the absence of salt. In fourteen pairs of duplicates evaporated from water, without alcohol extraction, the mean deviation from the mean was 2 per cent, no deviation exceeding 4 per cent. For testosterone in albumin or serum, duplicate values deviated from the mean by less than 4.5 per cent 17 out of 19 times. In fifteen pairs of duplicates, in which each sample was processed independently with its own standard, the mean deviation from the mean was 2.4 per cent for progesterone in water, no value deviating more than 5 per cent. In salt



solution, the mean deviation from the mean was 5 per cent for thirty pairs of determinations, 90 per cent of the values agreeing within 8 per cent. For progesterone in albumin or serum solution, duplicate values deviated from the mean less than 6 per cent 16 out of 22 times.

### Results

*Testosterone*—At 37.5°, all values for the solubility in water deviate from the mean of  $1.26 \times 10^{-4}$  moles per liter by less than 5 per cent, the standard deviation of the mean being 0.01. They, therefore, all agree within the experimental error. Change of solvent, as well as variation in concentration of solute, is without influence, confirming the purity of the solute. In the experiments in which equilibration was maintained for 140 hours, the melting point of the solute is unchanged. In the experiment in which equilibration was carried out for 319 hours, the melting point is lowered 0.5°, the solubility remaining the same. In general, the agreement for each solvent of the solubility values in the various salt concentrations confirms the agreement of results in water, the deviation being slightly greater. Only one value for 3.89 per cent NaCl, which deviates 19 per cent from the mean of the other values, is obviously out of line. The results are given in Table I.

At 25.0°, the four values for water obtained by supersaturation are in excellent agreement. The undersaturation values in 1.0 and 3.89 per cent NaCl are 10 per cent less than the value obtained from supersaturation, but the deviation approaches the experimental error.

In order to be certain that the product in solution was testosterone and not some decomposition product, an aqueous saturated solution, which had been subjected to equilibration over 140 hours, was evaporated to dryness *in vacuo* at room temperature over  $\text{CaCl}_2$ . The melting point of the residue was 153.4–154.2°, indicating pure testosterone.

The results recorded in Table I indicate that saturation of the solute is attained in a comparatively short period (70 hours). Before this was realized, preliminary experiments, in which equilibration was carried out over 500 hours, gave erratic results (20 per cent higher values) and it was found that when the result was erratic the melting point of the solute had suffered an appreciable lowering, 3.0°. Under these conditions, the results had no significance as to solubility. In the case of solubility in 3.89 per cent NaCl, the melting point of the solute was depressed only 0.5° after this long period of equilibration, the results agreeing with those obtained on shorter equilibration.

The solubility determinations in water and sodium chloride solutions were from unbuffered solutions. The pH of these solutions varied from 6.1 to 7.0. The influence of pH was studied by equilibration in 0.1 per

TABLE I  
*Solubility of Testosterone in Aqueous NaCl and Na<sub>2</sub>SO<sub>4</sub> Solutions*

Concentration of solute per 100 cc.	Equilibration		Solubility, moles per liter $\times 10^4$			
	Temperature	Time	Water	1 gm. NaCl per 100 cc.	2 gm. NaCl per 100 cc.	4 gm. NaCl per 100 cc.
mg.	°C.	hrs.				
20 (1)	37.5	70	1.25 (8)			
20 (1)	37.5	140	1.29 (4) (m.p. 154.0)			
10 (1)	37.5	70	1.24 (2)	1.17 (2)	1.09 (2)	0.82 (2)
10 (1)	37.5	140	1.20 (2) " (m.p. 154.0)	1.07 (2) (m.p. 154.0)	1.04 (2) (m.p. 154.0)	0.88 (2) (m.p. 154.0)
40 (1)	37.5	70	1.28 (2)	1.16 (2)	0.98 (2)	0.83 (2)
40 (1)	37.5	140	1.24 (2) (m.p. 154.0)	1.11 (2) (m.p. 154.1)	1.07 (2) (m.p. 154.0)	0.81 (2) (m.p. 154.1)
20 (1)	37.5	64	1.26 (1)	1.16 (1)	1.04 (1)	0.86 (1)
20 (2)	37.5	78	1.26 (1)	1.12 (1)	0.97 (1)	0.83 (1)
20 (3)	37.5	113	1.28 (1)	1.11 (1)	1.03 (1)	1.00 (1)
20 (4)	37.5	64	1.24 (1)* (m.p. 153.5)	1.14 (1)* (m.p. 153.0)	1.05 (1)* (m.p. 153.5)	0.81 (1)* (m.p. 153.5)
35 (1)	37.5	264	1.30 (1)	1.17 (1)	1.05 (1)	0.78 (1)
40 (1)	25.0	88	0.99 (1)*			
40 (1)	25.0	91	0.91 (1)*			
40 (1)	25.0	96	0.97 (1)*			
40 (1)	25.0	187	0.92 (1)* (m.p. 153.5)			
20 (1)	25.0	72		0.76 (2) (m.p. 154.0)	0.67 (2) (m.p. 154.0)	0.54 (2) (m.p. 154.0)
20 (2)	25.0	141		0.74 (3) (m.p. 154.0)	0.74 (3) (m.p. 153.8)	0.54 (3) (m.p. 154.0)
20 (3)	25.0	71		0.84 (3)* (m.p. 154.0)	0.76 (3)* (m.p. 154.0)	0.61 (3)* (m.p. 154.0)
6 (3)	25.0	140				0.63 (4)* (m.p. 153.5)
			0.1 per cent NaHCO <sub>3</sub> , pH 9.1, $\mu = 0.01$		2 gm. Na <sub>2</sub> SO <sub>4</sub> per 100 cc.	4 gm. Na <sub>2</sub> SO <sub>4</sub> per 100 cc.
20 (1)	37.5	70	1.21 (2)		0.92 (2)	0.66 (2)
20 (1)	37.5	140	1.23 (2)		0.88 (2)	0.66 (2)
20 (1)	37.5	70			0.90 (2)	0.75 (2)
20 (1)	37.5	140			0.91 (2) (m.p. 154.0)	0.76 (2) (m.p. 154.0)

The figures in parentheses following concentration of solute values (first column) indicate change of solvent; viz., (1) indicates original solvent, (2) indicates the second change, (3) indicates the third change of solvent, etc.

The figures in parentheses following solubility values indicate the number of analytical determinations. Unless noted by \* the values are obtained from undersaturation. When the approach was from supersaturation, the solute and solvent were preequilibrated 2 to 4 hours at 55–60°. The values obtained from supersaturation are noted by \*.

cent  $\text{NaHCO}_3$ . The pH of this solution (which had been shaken to reach pH equilibrium) was 9.1. The solubility value obtained,  $1.23 \times 10^{-4}$  mole per liter, agrees well with the value of  $1.26 \times 10^{-4}$  mole per liter obtained for water. The pH is obviously without influence.

*Progesterone, Water, and Sodium Chloride at 37.5°*—In contrast to testosterone, equilibrium with progesterone was not attained at 70 hours equilibration but was reached at 140 hours. Values attained from undersaturation at 140 or more hours and from supersaturation agree within 6 per cent (nine experiments) for solubility in water, *viz.*,  $(4.26 \pm 0.05) \times 10^{-5}$  mole per liter. Neither change of solvent nor a 4-fold change in solute affected the results. In salt solution, the agreement is subject to a greater variation, approximately double that found for water. This is in line with the results obtained with testosterone and indicates the error introduced by the extraction procedure. At pH 9.1 the solubility in 0.1 per cent  $\text{NaHCO}_3$  solution was  $3.7 \times 10^{-5}$  mole per liter. Melting point determinations of the solute after equilibration were performed in all experiments and the results in all cases were the same as that for the original solute or  $0.2^\circ$  to an extreme value of  $0.5^\circ$  higher. To demonstrate that the dissolved portion had not undergone decomposition, 350 cc. of solvent were separated from the insoluble solute phase and evaporated to dryness at room temperature *in vacuo*. One sample had been equilibrated at  $60^\circ$  for 3 hours; the other at  $37.5^\circ$  for 80 hours. The melting point of the residue from the latter experiment was  $127.5\text{--}128.5^\circ$  (corrected), that of the former  $127.3\text{--}128.3^\circ$  (corrected). The results are given in Table II.

*Progesterone, Water, and Sodium Chloride at 25.0°*—In preliminary experiments the solubility of progesterone at  $25.0^\circ$  was determined from supersaturation. The results were paradoxical in that the difference in different experiments was greater than the experimental error, duplicate analyses of the same experiment checking within 10 per cent. It was finally realized by studying the solubility both from undersaturation and supersaturation that there were two equilibrium forms of progesterone at  $25.0^\circ$ . These experiments were performed in a cabinet with violent agitation by a mechanical shaker. Under these conditions the solute phase recrystallized. In the experiments recorded graphically in Fig. 1, the solubility in salt solution from supersaturation reaches a low value at 165 hours, increasing again at 286 hours. These low values are considerably lower than the high value obtained from undersaturation. The high values obtained at 100 hours from undersaturation are not maintained in these cases on further equilibration. It was noted that even when approached from undersaturation a crystalline deposit of progesterone formed on the container at the junction of the surface layer with the container. It was concluded that when the low solubility was attained the solution was in equilibrium

with these new crystals; that when the high solubility was attained, equilibrium was controlled by the initial solid phase. It will be noted that in the case of water the low value from supersaturation was not attained

TABLE II  
*Solubility of Progesterone in Aqueous Salt Solutions*

Initial concentration of solute per 100 cc. solvent	Equilibration time	Condition to produce supersaturation	Solubility, moles per liter $\times 10^3$			
			Water	1 gm. NaCl per 100 cc.	2 gm. NaCl per 100 cc.	4 gm. NaCl per 100 cc.
mg.	hrs.					
40 (1)	300	None	4.2 (2)	4.15 (2)	3.35 (2)	3.15 (2)
40 (1)	600	"		4.2 (1)	3.5 (1)	3.1 (1)
40 (2)	300	"	4.35 (2)	3.7 (2)	3.3 (2)	2.45 (2)
40 (2)	600	"	4.4 (1)	3.6 (1)	3.1 (1)	2.7 (1)
40 (1)	70	"	3.5 (2)	3.0 (2)	2.5 (2)	2.2 (2)
40 (1)	140	"	4.0 (2)	3.5 (2)	3.4 (2)	2.9 (2)
10 (1)	70	"	3.5 (2)	3.0 (2)	2.3 (2)	2.0 (2)
10 (1)	140	"	4.2 (2)	3.3 (2)	2.9 (2)	2.5 (2)
10 (1)		3 hrs., 49-52°	4.6 (2)			
10 (1)	70	3 " 49-52°	4.3 (1)			
10 (1)	140	3 " 49-52°	4.3 (2)			
10 (1)	70	2.5 hrs., 60°	4.3 (1)			
10 (2)	70	19 hrs., 60°		3.7 (1)	3.2 (1)	2.6 (1)
10 (1)	3	None	6.1 (2)*	5.1 (2)*	4.8 (2)*	3.3 (2)*
10 (1)	6	"	6.3 (2)*	5.1 (2)*	4.9 (2)*	4.1 (2)*
			0.1 per cent NaHCO <sub>3</sub> , pH 9.1		2 gm. Na <sub>2</sub> SO <sub>4</sub> per 100 cc.	4 gm. Na <sub>2</sub> SO <sub>4</sub> per 100 cc.
10 (1)	140	"			2.8 (4)	2.2 (4)
10 (1)	210	"			2.9 (2)	2.3 (2)
10 (1)	100	"	3.3 (2)			
10 (1)	170	"	3.7 (2)			

The figures in parentheses following concentration of solute values (first column) indicate change of solvent, *viz.*, (1) indicates original solvent, (2) indicates the second change, (3) indicates the third change of solvent, etc.

The figures in parentheses following the solubility value indicate the number of analytical determinations. All solubility data are for 37.5°, except those indicated by \*, which are for 59.5°. Solubility was approached from undersaturation unless preequilibrated at a higher temperature as indicated in the third column.

(Fig. 1). This experiment was repeated, but the solvent was separated from the solute at the higher temperature used to produce supersaturation. In this case the solute was in equilibration with the crystals which deposited from the supersaturated solution and the solubility reached a low value. The melting point of the crystals which deposited was 127.8-128.8° (cor-

rected). In the other experiments, both those in which equilibrium was attained from undersaturation and from supersaturation, the solute phase after equilibration showed no change in melting point or a slight  $0.2\text{--}0.3^\circ$  elevation.

With the highest value obtained from undersaturation and the lowest value obtained from supersaturation or by continued equilibration after crystal formation, two sets of solubility values which agree within the ex-

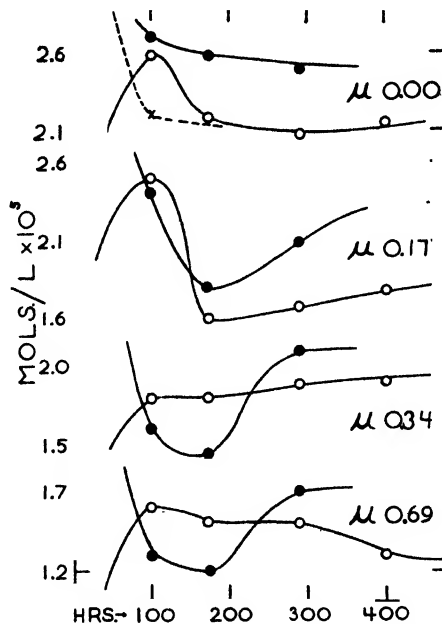


FIG. 1. The solubility of progesterone in water with increasing ionic strengths,  $\mu$ , of NaCl at  $25.0^\circ$ . ● indicates solubility values approached from supersaturation; ○ values approached from undersaturation; × indicates the experiment in which the solid phase of solute was removed after supersaturation.  $\mu$  is calculated per liter.

perimental analytical error with the Setschenow salting-out law are obtained.

Progesterone has two polymorphic modifications, one melting at  $128^\circ$ , the other at  $121^\circ$ . These have different crystalline forms and are readily interconvertible. However, from the melting points obtained in the foregoing experiments, the two equilibria at  $25.0^\circ$  cannot be explained on the basis of the interconversion of the form with a melting point of  $128^\circ$  to the form with a melting point of  $121^\circ$ . In the case of  $\text{As}_2\text{O}_3$ , three modifications, one of which is amorphous, have widely different solubilities in water. It is possible that progesterone has a third metastable form which

exists temporarily as crystallization at 25.0° proceeds from saturated solution. It should be borne in mind that, while progesterone has a readily producible melting point, it is not sharp, covering a range of about 1°, and this in itself would favor the existence of a polymorphic modification.

The solubility values obtained for both testosterone and progesterone in aqueous sodium sulfate solution are slightly lower than the values predicted from the solubility in aqueous sodium chloride solution on the basis

TABLE III

*Solubility of Testosterone in Bovine Albumin Solution at Ionic Strength  $\mu = 0.155$  with 1 Mg. of Solute per Cc. of Solvent*

Temperature	Per cent albumin	pH	Equilibration time	Solubility	M.p. of solute after equilibration
°C.			hrs.	moles per l. $\times 10^4$	°C.
37.5	3.0	5.3	6	10.9 (2)	153.5-154.0
		5.3	12	11.8 (2)	
		5.3	24	11.6 (6)	
		5.3	28	11.9 (2)	
		5.3	40	11.6 (2)	
37.5	3.0	7.4	23	14.2 (2)	153.0-153.5
		7.4	37	13.2 (2)	
37.5	3.0	8.0	30	15.6 (2)	153.3-153.8
		8.0	45	15.3 (2)	
37.5	1.0	5.4	24	5.4 (2)	153.3-154.0
		5.4	38	4.9 (2)	
25.0*	3.0		23	9.3 (2)	153.0-153.5
		5.3	32	9.0 (2)	
25.0	3.0	5.3	28	10.7 (2)	153.0-153.5
		5.3	52	10.6 (2)	
25.0	3.0	7.2	24	12.6 (2)	153.0-153.3
			48	12.3 (2)	

The figures in parentheses following solubility values indicate the number of analytical determinations.

\* Approached from supersaturation, after 40 hours equilibration at 37.5°.

of ionic strength, but the agreement is far closer than it is for solubilities of proteins in aqueous solutions of these salts (4).

*Solubility in Protein Solution*—The solubility data for testosterone in solution of crystalline albumin from bovine plasma indicate that equilibrium is rapidly reached. After 24 hours equilibration, the value lessens slightly in each experiment, although the difference individually is not significant. This difference may be explained on the basis of protein denaturation. Although pH had no effect upon testosterone solubility in aqueous solution in the physiologic range, pH has a pronounced effect in

albumin solution. In the range studied, pH 5.3 to 8.0, histidine is responsible for the change of base bound per mole of protein because of its ionization constant, and the increase in solubility may be related to this phenomenon. Although the solubility of testosterone in 3 per cent albumin at pH 7.4 is approximately 3.5 times that of progesterone, it is apparently significant that the increase in solubility with increase in pH for the range pH 5.3 to

TABLE IV

*Solubility of Progesterone in Bovine Albumin Solution at Ionic Strength  $\mu = 0.155$  with 0.4 to 0.75 Mg. of Solute per Cc. of Solvent*

Temperature	Per cent albumin	pH	Equilibration time	Solubility	M.p. of solute after equilibration
°C.			hrs.	moles per l. $\times 10^4$	°C.
37.5	3.0	5.3	6	2.7 (2)	128.0-128.7
			12	3.3 (2)	
			24	3.4 (6)	
			32	3.4 (2)	
37.5	3.0	7.4	24	3.8 (2)	127.8-128.5
			38	4.4 (2)	
37.5	3.0	8.1	30	6.2 (2)	127.7-128.6
			45	6.8 (2)	
37.5	1.0	5.3	25	1.3 (2)	127.3-127.5
			39	1.5 (1)	
25.0	3.0	5.3	24	2.1 (4)	128.0-129.0
			36	1.9 (2)	
			52	2.5 (4)	
			70	2.6 (2)	
			94	2.6 (2)	
25.0	3.0	7.1	48	3.0 (2)	127.7-128.7
			72	2.9 (2)	

The figures in parentheses following solubility values indicate the number of analytical determinations.

8.0 is virtually identical, *viz.*, 3.8 *versus*  $3.4 \times 10^{-4}$  mole per liter. The results are given in Table III.

In contrast to testosterone, the solubility for progesterone in 3.0 per cent albumin solution increased after 24 hours equilibration in four of the experiments. As was the case for aqueous solution, this indicates that equilibrium for progesterone is more slowly attained than for testosterone. See Table IV.

The solubility of testosterone in bovine  $\gamma$ -globulin was determined at ionic strength  $\mu = 0.155$  with 0.5 mg. of solute per cc. of solvent in 3.0 per cent protein solution at pH 7.2 and 37.5°. Values for the equilibration

times of 24 and 44 hours were identical; namely,  $2.6 \times 10^{-4}$  mole per liter. This compares with a value of  $14.2 \times 10^{-4}$  mole per liter for 3 per cent albumin at approximately the same pH. A value for the solubility of progesterone in  $\gamma$ -globulin under the same conditions could not be determined, as it virtually approached that for isotonic saline.

The solubility values for testosterone and progesterone in rabbit serum are given in Table V. There appears to be a rough correlation between protein content and solubility. It is apparent that albumin is responsible for approximately 70 per cent of the solubility of testosterone in serum and

TABLE V  
*Solubility of Testosterone and Progesterone in Rabbit Serum at 37.5°*

Steroid	Protein content of serum	pH	Equilibration time	Solubility
	<i>per cent</i>		<i>hrs.</i>	<i>moles per l. <math>\times 10^4</math></i>
testosterone	7.3	8.5	24	35 (6)
	7.3	8.5	46	31 (9)
	6.0	8.3	24	33 (6)
	5.8	8.2	20	28 (2)
progesterone	6.1	8.5	24	10.9 (4)
	6.1	8.5	44	12.5 (4)
	6.6	8.3	48	14.0 (4)
	6.6	8.2	72	14.1 (4)
	5.8	8.2	20	9.5 (2)
	6.2	8.3	24	11.8 (2)

In all cases the melting point of the solute after equilibration was within  $0.2$ – $0.5^\circ$  of that of the original substance. The figures in parentheses after the solubility values indicate the number of analytical determinations. 2.5 mg. of testosterone per cc. of solvent and 1.5 mg. of progesterone per cc. of solvent were used in all experiments.

for approximately 85 per cent of the solubility of progesterone in serum. The difference is probably relegated to globulin, which had no dispersing power toward progesterone. On the basis of published estrogen determinations (5) of human plasma fractions one might expect the  $\beta_1$ -lipoprotein fraction, which was found to contain most of the estrogen activity, to contribute to the steroid solubility. The balance sheet for albumin,  $\gamma$ -globulin, and lecithin (in the case of estradiol) in our studies leaves little room for the  $\beta_1$ -lipoprotein fraction. The estrogen reported in the  $\beta_1$ -lipoprotein fraction may be esterified, and hence manifest markedly different physical properties. This question is at present under investigation. At any rate the estrogen is reported not to be estradiol.



## DISCUSSION

The decrease in solubility produced by electrolytes was first described in terms of an exponential equation, empirically derived by Setschenow from solubility data of gases in salt solution (6).

$$\ln S = \ln S_0 - KC \quad (1)$$

in which  $S_0$  is the theoretical solubility in water,  $S$  the solubility in the electrolyte solution,  $C$  the concentration of electrolyte, and  $K$  a constant. Debye and MacAuley (7) described the activity coefficients of the solute in salt solution, which are measured by the deviation in solubility, in terms of an equation in which activity is roughly proportional to electrolyte concentration. Hückel (8) added to this equation a correction for the change in the dielectric constant of the solution with electrolyte concentration. The equation describing the effect of the electrolyte on the non-electrolyte solubility is,

$$\ln \gamma_n = (Ne^2 \zeta_+ \zeta_- \delta_n / 2RTb_i D_0^2 p_0) vm_i \quad (2)$$

in which  $\gamma_n$  is the activity coefficient of the non-electrolyte,  $N$  is Avogadro's number,  $e$  is the proton charge,  $\zeta$  the valence,  $\delta_n$  the molar dielectric increment,  $b_i$  the radius of the salt ion,  $D_0$  the dielectric constant of the solvent,  $p_0$  the polarization of the solvent,  $v$  the number of cations and anions, and  $m_i$  the moles per kilo of electrolyte. The salting-out effect depends upon the temperature, upon the dielectric constant of the solvent, upon the rate of change of dielectric constant with changing concentration of the solute, and upon the valence and radii of the ions.

If the original Setschenow equation holds, these effects are incorporated in its two constants, in which  $S_0$  is dependent upon temperature and  $K$  combines the salting-out effect and salting-in effect.  $K$  holds when the salting-out effect is large compared with the salting-in effect.

The values of the constants of the Setschenow equations depend upon whether solubility is expressed as the mole fraction, moles per liter, moles per kilo of solvent, or moles per kilo of water and whether concentration is expressed as  $\mu$ , the ionic strength of Lewis (9) calculated per kilo of water or that of Debye calculated per liter of solvent. While mathematically the equation could not hold equally well for any of these expressions, experimentally the accuracy of fit has been found to be of about the same order. With the Debye concept, our data as treated in Fig. 2 are based upon the equation

$$pS = pS_0 + K(r/2) \quad (1, a)$$

in which  $pS$  is the negative logarithm of the solubility in moles per liter,  $pS_0$  is the negative logarithm of the solubility in water in moles per liter,

and  $\Gamma$  is the ionality (double ionic strength) in moles per liter.  $\Gamma = \sum C\zeta^2$  in which  $C$  is the concentration of each ion and  $\zeta$  is the valence of the ion.

Frequently the expression  $S_0$  in the Setschenow equation is replaced by a constant  $B$ , particularly when the actual solubility in water is not equal to the value  $S_0$ , which is regarded as a hypothetical solubility obtained by extrapolation.  $S_0$  may be more than a constant empirically determined, since under certain conditions it becomes one of the parameters in determining the activity coefficient of the solute, viz.,  $\gamma = S_0/S^1$ , where  $\gamma$  is the activity coefficient,  $S_0$  is the solubility derived from the Setschenow equation, and  $S^1$  is the actual solubility.

Since the activity of the solid solute is taken as 1,  $S^1\gamma/1 = k$ , which is the mass law in its simplest form. In the Setschenow equation, when  $S^1$  is negligible compared with the concentration of salting-out solute,  $S_0$  becomes the solubility at infinite dilution of both solutes.  $S_0$  then equals  $k$ , since the activity coefficient at infinite dilution is 1, and finally

$$S^1\gamma = S_0 \quad (3)$$

Obviously,  $S_0$  in the Setschenow equation becomes valid for the determination of the activity coefficient only if the experimental values give a linear extrapolation,<sup>1</sup> which in itself is a test of the law.

These conditions have been met in the data presented graphically in Fig. 2.

If we substitute the solubility values as negative logarithms in the Setschenow equation and take the intercept at 0 ionic strength as  $S_0$  (or  $B$ ), the value for  $+K$  for testosterone in NaCl at 37.5° becomes 0.27; for NaCl at 25° it becomes 0.28, and for Na<sub>2</sub>SO<sub>4</sub> at 37.5°, 0.31. The intercept at zero ionic strength agrees with the value found experimentally in water well within the experimental error, showing that within this error the activity coefficient of testosterone is 1.0. The values of  $+K$  for progesterone are 0.32 for NaCl at 37.5° and 0.35 for sodium sulfate. The intercept at zero ionic strength agrees well with the experimental value obtained in water, indicating an activity coefficient of 1.0 for progesterone. It will be noted that the values of  $K$  for progesterone are in fairly close agreement with those obtained for testosterone, indicating a marked similarity in the response to salting-out, although the absolute solubility values for progesterone are about one-third the values for testosterone.

Included in Fig. 2 are published (10) and unpublished data for the solubility of estradiol in aqueous solution at varying ionic strengths in the range pH 6.5 to 8.0. These values were obtained by bioassay, with an

<sup>1</sup> When  $S$  is not small compared to the concentration of salting-out solute,  $\log S$  is plotted against  $S + S_{\text{salt}}$  or  $\sqrt{S + S_{\text{salt}}}$  to obtain a linear extrapolation giving the solubility at infinite dilution for both solutes.

accuracy within 15 per cent 19 out of 20 times (11). The agreement of the available data with the Setschenow equation is realized within the error of the assay.  $+K$  in the Setschenow equation becomes approximately 0.20. In the pH range studied pH is without effect upon solubility (10), indicating complete hydrolysis, which is consistent with an ionization constant as weak as that of phenol. Moreover, the ionization would be less than 0.1 per

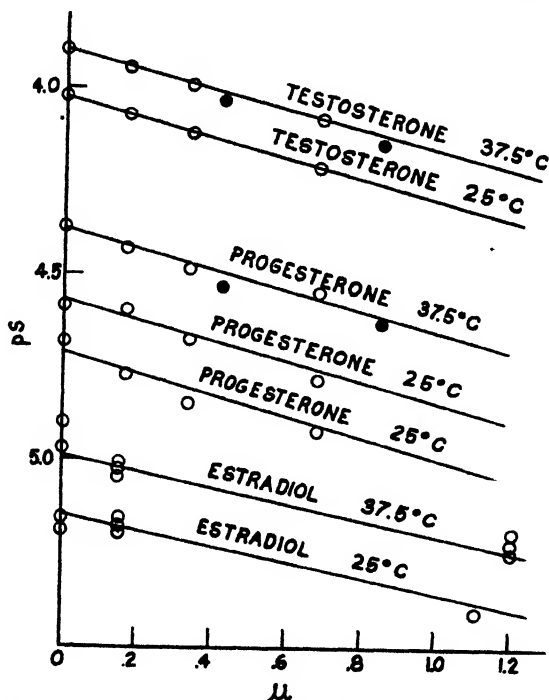


FIG. 2. The solubility of testosterone, progesterone, and  $\alpha$ -estradiol in aqueous solutions of varying ionic strength.  $pS$  is the negative logarithm of the solubility in moles per liter.  $\mu$  is the ionic strength of the electrolyte.  $\circ$  indicates  $NaCl$  as the electrolyte;  $\bullet$  indicates  $Na_2SO_4$  as the electrolyte.  $\mu$  is calculated per liter.

cent; so that the estradiol in solution behaves substantially as a non-electrolyte.

Since under the conditions of our solubilities of testosterone, progesterone, and estradiol, the ratio of the solubility in water to that in aqueous salt gives the activity coefficient,

$$\ln \gamma_n = \ln S_0 - \ln S = (N_0^2 \epsilon_+ \epsilon_- \delta_n / 2RTb_+ D_0^2 p_n) v m, \quad (1, 2, 3)$$

Now, since  $\ln S_0 - \ln S = KC$ , the salting-out constant in the Setschenow equation becomes equal to the expression in parentheses in equation (2). In comparing the solubilities of the three steroids at the same temperature

and in the same ionic environment, all the parameters in equation (2) are constant except  $\delta_n$ , which is proportional to  $K$  in the Setschenow equation. The molar dielectric increment of estradiol is, therefore, about two-thirds that of testosterone or progesterone. These constants would in all cases be negative. Since the molecular model assumed in equation (2) is oversimplified, calculated values for the molar dielectric increments could not be considered too seriously, but the order of magnitude should be correct, however.

Since albumin is in colloidal solution, and since the other constituents of serum which contribute to the dispersion of the steroids are also in colloidal solution, the question arises as to whether the steroid is adsorbed by the colloid or whether it is oriented in the solvent by secondary valence bonds of the colloid. The distinction is probably one of degree, but we are more inclined to hold that it is not adsorption, because on the introduction of another phase (10) the steroid is in equilibrium with the second phase as though it were in true solution. It is interesting to compare the relative dispersing powers of albumin toward the three steroids. At pH 7.0 to 7.4 estradiol is 22 times as soluble in 3 per cent albumin solution as in isotonic saline, but this ratio is only 12 to 13 times for testosterone or progesterone. Testosterone is about 3 times as soluble in isotonic saline as progesterone. This ratio holds roughly for solubility in albumin in the range pH 5.3 to 8.0 and in serum at pH 8.3 to 8.5. A comparison of the dispersing power of  $\gamma$ -globulin with that of albumin for the steroids reveals that for estradiol the globulin is 40 per cent as effective as albumin, but only 12 per cent as effective for testosterone. The dispersing power of globulin for progesterone is nil. The solubility of estradiol in 1 per cent isotonic  $\gamma$ -globulin solution at pH 7.0 was  $3.9 \times 10^{-5}$  mole per liter. The weak dispersing power of  $\gamma$ -globulin compared with albumin, as regards the steroid hormones, is probably of physiologic significance, emphasizing the unique qualities of albumin in binding substances with the polar properties of the steroids.

A comparison of the dispersion of testosterone, progesterone, and estradiol in 1 and 3 per cent albumin solution was made at 37.5° at the same pH. When solubility in non-protein isotonic salt solution is subtracted, the dispersion of estradiol is proportional to the protein concentration. For both testosterone and progesterone, the ratios are 1:2.7 instead of 1:3.0. Considering the possibility of analytical errors of 5 per cent, the agreement is reasonably close. On a molar basis albumin disperses about 0.5 mole of estradiol, 0.7 to 1.5 moles (depending on pH) of progesterone, and 2.5 to 3.3 moles (depending on pH) of testosterone. 1 mole of fatty acid is contained per mole of crystalline albumin and is regarded as necessary to achieve crystallization.

The heats of solution or dispersion calculated from the van't Hoff re-

action isochor are given in Table VI. The number accompanying each heat of solution is either the standard deviation of the mean or an approximation of this constant. For progesterone in water or aqueous salt solution the equilibrium form having the highest solubility was used in the calculation, since the evidence at hand indicated that the solute was in equilibrium with the original solid phase. In calculating the heat of dispersion in protein solution, the solubility in isotonic saline is subtracted from each solubility value before the calculation is made. This value is, therefore, not a heat of solution, since the disperse phase is in equilibrium not with the solid phase but with the aqueous non-protein electrolyte phase. The heat of dispersion is, therefore, the heat involved in the orientation of the steroid by the protein. The heat of dispersion is presented as a new con-

TABLE VI

*Heats of Solution and Dispersion of Testosterone, Progesterone, and Estradiol in Water, Aqueous NaCl, or 3 Per Cent Bovine Albumin Solutions*

Solute	Solvent	Heat of solution (or dispersion)
		<i>calories per mole</i>
Testosterone	Water	4,300 $\pm$ 100
	1, 2, or 4% NaCl	4,400 $\pm$ 100
	3% albumin, pH 5.3	1,100 $\pm$ 400
	3% " " 7.3	1,900 $\pm$ 900
Progesterone	Water	7,300 $\pm$ 1300
	1, 2, or 4% NaCl	6,700 $\pm$ 1000
	3% albumin, pH 5.3	3,600 $\pm$ 1200
	3% " " 7.3	5,500 $\pm$ 1100
Estradiol	0.9% NaCl	4,300 $\pm$ 1400
	3% albumin, pH 5.3	13,700 $\pm$ 2000

cept, following logically from (a) the temperature coefficients of dispersion and (b) the concentration of steroid by a protein solution phase separated by a membrane from an aqueous non-colloidal phase (10). The heat of dispersion of estradiol by albumin is high compared with that of either testosterone or progesterone and is further characterized by its independence of pH.

Since testosterone and progesterone were determined by the Zimmermann reaction which does not respond to estradiol and since estradiol was determined by an assay which was not sensitive to the amounts of testosterone or progesterone present, it was possible to determine the mutual effect of two steroids on their dispersion by albumin solution. This experiment is reported in another paper (10), but the results should be considered in summarizing the effect of dispersion by protein. While estradiol had no measurable effect upon the dispersion of the two other steroids, its dis-

persion was significantly depressed by the other two. The influence which estradiol would be expected to have on the two other steroids is probably within the experimental error; since the concentration of estradiol is so much less than that of the other steroids, its influence would not be measurable.

In summarizing, one is impressed by the characteristic differences not only in the two proteins studied, but also in the differences in response of the three steroids for the same protein. These differences are probably of fundamental importance in the distribution of the steroid in the organism.

#### SUMMARY

The solubilities of testosterone, progesterone, and  $\alpha$ -estradiol in aqueous sodium chloride or sodium sulfate solution in the temperature range 25–37.5° follow the Setschenow equation,  $pS = pS_0 + K\mu$ , in which  $pS$  is the negative logarithm of the solubility in moles per liter,  $pS_0$  is the negative logarithm of the solubility in water in moles per liter,  $K$  is the salting-out constant, and  $\mu$  is the ionic strength.  $pS_0$  determined by extrapolation agrees with the solubility in water experimentally determined within the error of the method used, and so the activity coefficients for solubility in water are 1.0 within that error. At 37.5°,  $pS_0$  is 3.90 for testosterone, 4.37 for progesterone, and 5.0 for estradiol.

Within the range pH 5 to 8, pH is without appreciable influence on the solubility of the three hormones in aqueous non-protein solution.

At 25°, progesterone solubility depended upon two equilibrium phases. Testosterone, m.p. 153.7–154.2° (corrected), and progesterone, m.p. 127.5–128.5° (corrected), gave the same respective solubilities with a 4-fold change in concentration or a 3-fold change in solute, indicating that these are the melting points for the pure compounds.

At pH 5.3 to 5.5 and 37.5°, the solubilities in isotonic solution containing 3.0 gm. per 100 cc. of bovine crystalline albumin were  $2.0 \times 10^{-4}$  mole per liter for estradiol,  $3.4 \times 10^{-4}$  mole per liter for progesterone, and  $11.8 \times 10^{-4}$  mole per liter for testosterone. An increase of pH from 5.3 to 8.0 increases the dispersion of both testosterone and progesterone by  $3.6 \times 10^{-4}$  mole per liter. The dispersion of estradiol is not affected by pH. Bovine  $\gamma$ -globulin at pH 7.0 had no dispersing power toward progesterone.

The heats of solution or dispersion in the temperature range 25.0–37.5° are calculated from the van't Hoff isochor. The heat of dispersion is introduced as a new concept.

We wish to thank Dr. Erwin Schwenk of the Schering Corporation for the steroid hormones used in these experiments and Dr. J. B. Lesh of Armour and Company for the  $\gamma$ -globulin.

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# FRACTIONATION, ELECTROPHORESIS, AND CHEMICAL STUDIES OF PROTEINS IN SERA OF CONTROL AND INJURED GOATS\*

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Cohn and his associates (1) introduced new and valuable procedures for fractionating human serum proteins by using varying concentrations of ethanol at different hydrogen ion concentrations, low ionic strengths, and low temperatures. These principles were utilized in fractionating the serum proteins of control and injured dogs (2) and rats (3). Dogs injured by turpentine, heat, or sulfur mustard show marked changes in the electrophoretic patterns of their sera and fractions, due in part to the appearance of new proteins and to increased amounts of lipoproteins. Since data concerning goat serum are not available, the present study was undertaken to determine (a) conditions for fractionation and (b) the distribution of proteins and their associated lipides in the serum of control and injured animals.

## Methods

The description of the apparatus used, methods for adjusting ethanol concentrations, ionic strength, pH, the electrophoretic technique, and the analytical procedures have been presented previously (2).

Small individual samples of goat serum were obtained by withdrawing blood from the external jugular vein. Large amounts of serum were obtained from the pooled blood of a number of goats which were exsanguinated under anesthesia. Serum was sent to this laboratory packed in dry ice. These sera were maintained at low temperature and were thawed at 5° just before being fractionated.

Groups of goats were gassed (head excluded) with the vapor of sulfur mustard, bis( $\beta$ -chloroethyl)sulfide (H) and by cutaneous application of 50 mg. per kilo of H to the shaved back. A number of goats were each injected subcutaneously with 0.5 ml. quantities of turpentine into ten

\* The work described in this paper was done under contract between the Medical Division, Chemical Corps, United States Army, and the University of Virginia. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

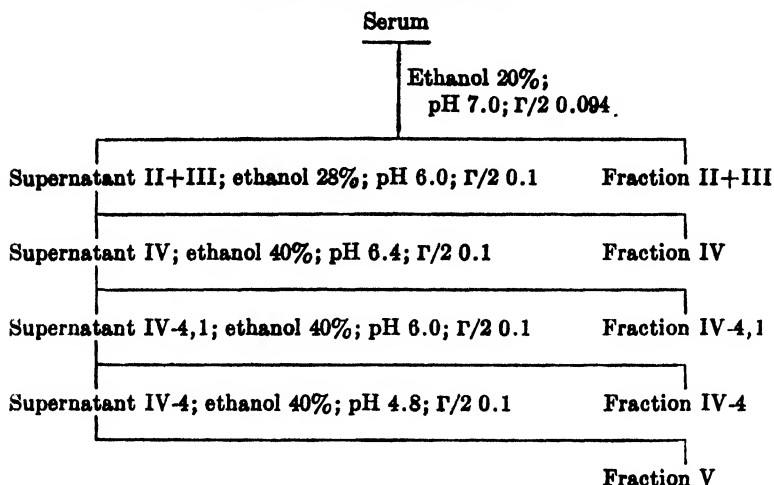


different sites. These animals were exsanguinated 7 days after injection and the blood was pooled. Third degree burns on about 20 per cent of the body area were produced in other animals.

### *Fractionation Procedures*

The first procedure used for fractionation was that recommended for human serum (1); the results were unsatisfactory except for fraction II+III. Fractions IV and IV-4 contained practically all of the albumin

DIAGRAM 1  
*Major Fractionation of Serum\**



\* The details of the fractionation procedure may be obtained, upon request, from this laboratory.

in addition to the  $\alpha_1$ - and  $\alpha_2$ -globulins. The yield of fraction V was negligible.

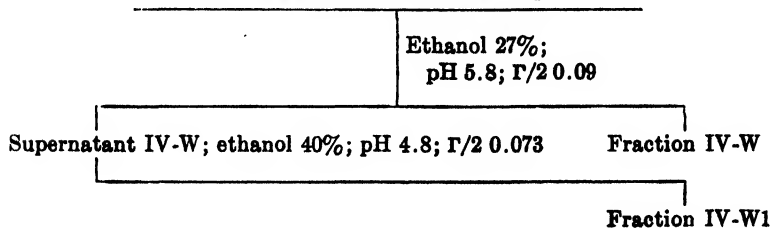
A second procedure was developed after studying the effect of pH, ethanol concentration, ionic strength, and the type of salt on the separation of the components of the fractions IV and IV-4. It was found that relatively high concentrations of sodium chloride were more effective than sodium acetate in decreasing the albumin content of these two fractions. It was also noted that the  $\alpha$ -globulins were effectively precipitated at relatively high pH and ethanol concentrations. The procedures outlined in Diagrams 1 to 4 were used for obtaining most of the data presented in this paper. Five main fractions could be obtained (Diagram 1) but in most cases fractions IV-4,1 and IV-4 were combined by adjusting the supernatant of fraction IV to pH 6.0 and 40 per cent ethanol.

Further experimental work was undertaken to improve the fractionation of IV-4 (includes IV-4,1) since this fraction yielded extremely turbid solutions after short periods of storage in the cold, which appeared to be

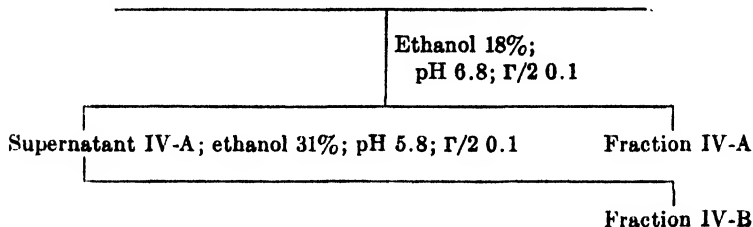
### DIAGRAM 2

#### *Subfractionation of Fraction IV*

Fraction IV dissolved in 5 times its weight of H<sub>2</sub>O



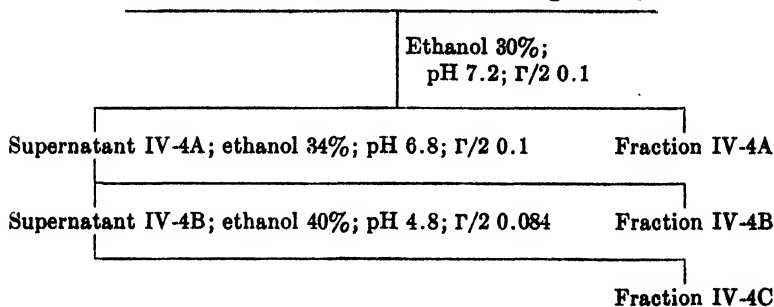
Dissolve Fraction IV-W in 6 times its weight of H<sub>2</sub>O



### DIAGRAM 3

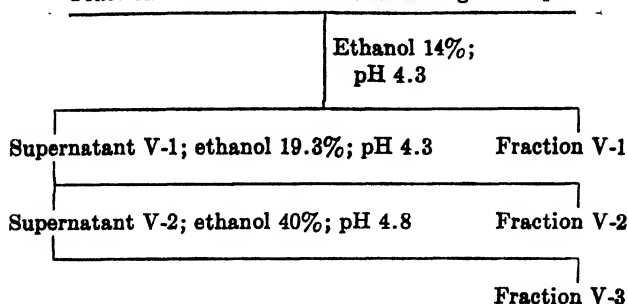
#### *Subfractionation of Fractions IV-4 and IV-4,1*

Fraction IV-4 dissolved in 20 times its weight of H<sub>2</sub>O



due to denaturation of  $\alpha_1$ -globulin. It was found that the components of fractions IV-4 and V could be precipitated from supernatant IV at 30 per cent ethanol, pH 4.8, and ionic strength of 0.09 (Diagram 5). After raising the ethanol concentration of the supernatant to 40 per cent, a small precip-

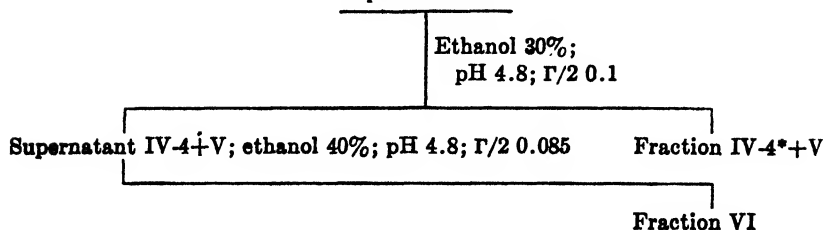
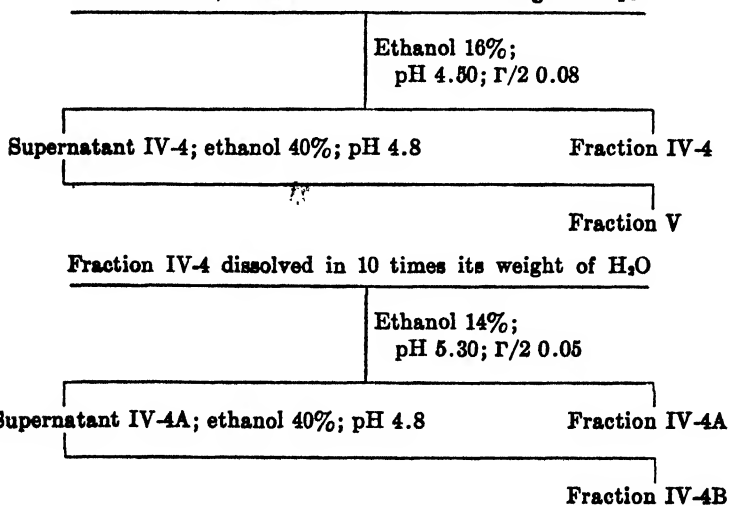
## DIAGRAM 4

*Subfractionation of Fraction V*Fraction V dissolved in 6 times its weight of H<sub>2</sub>O

## DIAGRAM 5

*Modification in Major Fractionation of Goat Serum*

Supernatant IV

*Modified Subfractionation of Fractions IV-4+V*Fraction IV-4+V dissolved in 10 times its weight of H<sub>2</sub>O

\* Includes IV-4 1

itate, consisting of  $\alpha_1$ -globulin, appeared (fraction VI) which yielded a very turbid solution. The fraction IV-4+V yielded a clear solution.

Experiments were conducted to determine the best conditions for sub-fractionation of IV-4+V. It was found that the globulins could be separated from albumin most effectively at (1) a pH on the acid side of the isoelectric point of albumin, (2) low ethanol concentrations, and (3) a relatively high ionic concentration maintained with sodium acetate. High  $\alpha_1$  and comparatively low albumin concentrations could be attained for fraction IV-4 without denaturation of the  $\alpha_1$ -globulin.

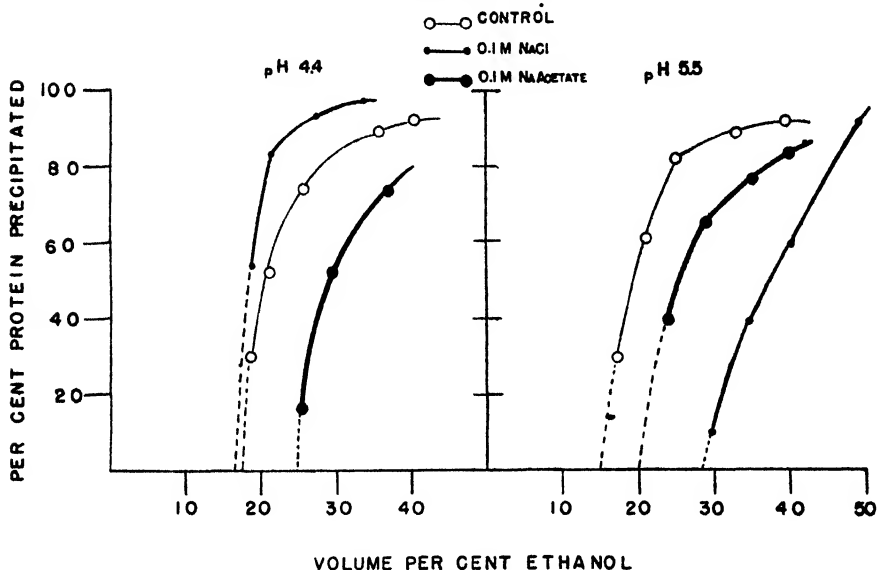


FIG. 1. Influence of sodium chloride and sodium acetate on the solubility of electrophoretically pure goat albumin in the presence of varying concentrations of ethanol.

In order to determine the effects of 0.1 M sodium chloride or sodium acetate at pH values below and above the isoelectric point at varying concentrations of ethanol, the solubility of electrophoretically pure goat albumin was studied. The albumin solution (1.0 per cent) was dialyzed at 5° for 4 days against frequent changes of distilled water. Aliquots, which were adjusted to pH 4.4 and 5.5 with a minimum of sodium acetate in acetic acid buffer to give a final ionic strength of 0.005, served as "salt-free" controls. Other aliquots were adjusted to the desired pH, ionic strength (0.1), and ethanol concentration by adding calculated amounts of sodium chloride or sodium acetate and buffers dissolved in 53.3 per cent ethanol. The precipitates formed at different ethanol concentrations were analyzed for nitrogen and the results plotted (Fig. 1). The solubility

of albumin was increased by sodium acetate and especially by sodium chloride at a pH above its isoelectric point. On the other hand, at pH 4.4 sodium acetate increased and sodium chloride decreased the solubility of albumin. These data were useful in developing the procedures for separating albumin from globulins as shown in the subfractionation procedures (Diagram 5).

### Results

*Controls*—A number of sera of control goats were analyzed electrophoretically. The mean values and standard deviations for the mobility

TABLE I  
*Mobilities\* and Percentage Distribution of Components in Control Goat Serum*

Boundaries	Albumin	Globulins			
		$\alpha_1$	$\alpha_2$	$\beta$	$\gamma$
Mobilities					
Descending (7 goats)	6.9 $\pm$ 0.05	5.3 $\pm$ 0.04	4.0 $\pm$ 0.04	3.0 $\pm$ 0.03	1.8 $\pm$ 0.03
Ascending (10 goats)	7.5 $\pm$ 0.05	6.0 $\pm$ 0.04	4.7 $\pm$ 0.05	3.6 $\pm$ 0.03	2.3 $\pm$ 0.03
Per cent distribution					
Descending (10 goats)	37 $\pm$ 2.5	11 $\pm$ 0.8	13 $\pm$ 0.8	8 $\pm$ 1.4	31 $\pm$ 0.6
Ascending (11 goats)	37 $\pm$ 2.7	10 $\pm$ 0.9	12 $\pm$ 0.6	8 $\pm$ 1.0	33 $\pm$ 0.6

\* In barbital buffer at pH 8.6, ionic strength 0.1, and temperature 2° expressed as cm.<sup>2</sup> volt<sup>-1</sup> sec.<sup>-1</sup>  $\times 10^{-5}$ . Serum diluted 1:1.5.

of each protein component are shown in Table I. This information served as a guide for identifying the components in each fraction. The values for the percentage distribution of the protein components served as a measure of the degree of variation for control animals. Electrophoretic patterns and analyses for a number of fractions are shown in Fig. 2.

The electrophoretic patterns of goat serum consistently yield five distinct components. The resolutions of the boundaries are superior to those encountered in the dog and rat. The fractions and subfractions of goat serum also yield patterns which are well resolved.

Deutsch and Goodloe (4) have presented data for the mobility and percentage distribution of the protein components of a single goat plasma. Their data for mobilities of the descending limbs are corroborated by the result shown in Table I. The sera of the goats used in the present experi-

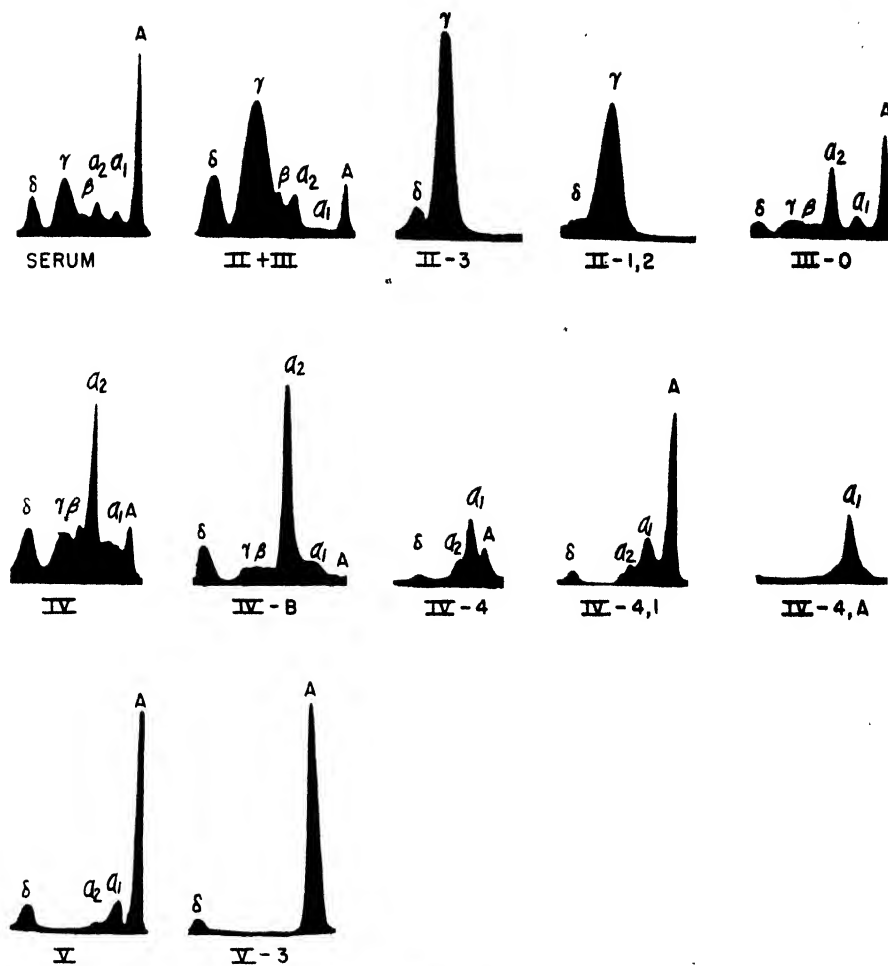


FIG. 2. Electrophoretic patterns and analyses of control goat serum and its fractions.

Fraction	Per cent distribution					Fraction	Per cent distribution				
	Albu- min	$\alpha_1$	$\alpha_2$	$\beta$	$\gamma$		Albu- min	$\alpha_1$	$\alpha_2$	$\beta$	$\gamma$
Serum	39	9	11	6	35	IV-4	30	58	12		
II+III	6	3	11	8	72	IV-4,1	65	25	10		
II-3					100	IV-4A	6		86	8	
II-1,2					100	V	79	17	4		
III-0	37	12	30	5	16	V-3	100				
IV	12	15	40	10	23						
IV-B	3	12	70	6	9						

ments contained greater concentrations of  $\gamma$ -globulins and less albumin than those reported by the Wisconsin investigators.

*Fraction II+III* contains practically all the  $\gamma$ - and  $\beta$ -globulins in the serum as well as appreciable amounts of  $\alpha$ -globulin and albumin. Subfractions II-1,2 and II-3 can be obtained as electrophoretically pure  $\gamma$ -globulins. The mean values for the mobilities for II-1,2 are 1.37 and  $1.5 \times 10^{-5}$  for descending and ascending boundaries, respectively; corresponding values for II-3 are 1.69 and  $2.0 \times 10^{-5}$ . For further proof of the presence of two distinct globulins, a mixture of equal amounts of II-1,2 and II-3, subjected to electrophoresis for 5 hours at a potential gradient of 6 volts, yielded two distinct components. These two components appear to correspond to the  $\gamma_1$ - and  $\gamma_2$ -globulins described for human sera by Duetsch *et al.* (5). Subfraction III-1 consists of  $\gamma$ -,  $\beta$ -, and  $\alpha_2$ -globulins. Fraction III-3 is composed chiefly of  $\gamma$ -globulins. Fraction III-0 contains all of the albumin and  $\alpha_1$ -globulin present in II+III and in addition is rich in  $\alpha_2$ - and  $\beta$ -globulins. Fraction III-2 always yields solutions which are too turbid for electrophoresis. Fractions II-3 and II-1,2 could be lyophilized without affecting their solubilities in saline; fractions III-3 and III-2 become insoluble under the same treatment.

Mobility values for the  $\gamma$ -globulin in the subfractions of II+III are invariably greater (range 2.0 to  $3.2 \times 10^{-5}$ ) than those obtained for the electrophoretically pure II-3 and II-1,2 components, which may be due to interaction between proteins.

*Fraction IV* is obtained in small amounts and is rich in  $\alpha_2$ -globulin. By subfractionation, it is possible to concentrate the  $\alpha_2$ -globulin in IV-B and the albumin in IV-W,1.

The main fractions IV-4 and IV-4,1 are composed chiefly of  $\alpha_1$ -globulin and albumin. Since they are obtained in small amounts, they are combined for subfractionation according to Diagram 3. An unusually high concentration of  $\alpha_1$ -globulin is present in IV-4A. Fraction IV-4B is composed chiefly of  $\alpha_1$ - and  $\alpha_2$ -globulins and IV-4C contains most of the albumin of the main fraction. If these three subfractions are allowed to stand at low temperatures, the  $\alpha_1$ -globulins become denatured and insoluble.

*Fraction V* is composed chiefly of albumin. By subfractionation it is possible to obtain an electrophoretically pure albumin (V-3). Fraction V-1 contains most of the  $\alpha_1$ -globulin, which is responsible for the marked turbidity when dissolved. Fraction V-2 contains a small amount of  $\alpha_1$ -globulin.

Yields of major fractions and subfractions of control serum were determined in terms of dry weight and as nitrogen. Fractions II+III and V are present in greatest amounts. The two pure  $\gamma$ -globulins, II-1,2 and

II-3, comprise a large part of fraction II+III. About one-half of the total albumin in fraction V could be obtained in pure form.

*Turpentine and Sulfur Mustard*—Electrophoretic studies of the whole sera of goats treated with turpentine and sulfur mustard showed that the

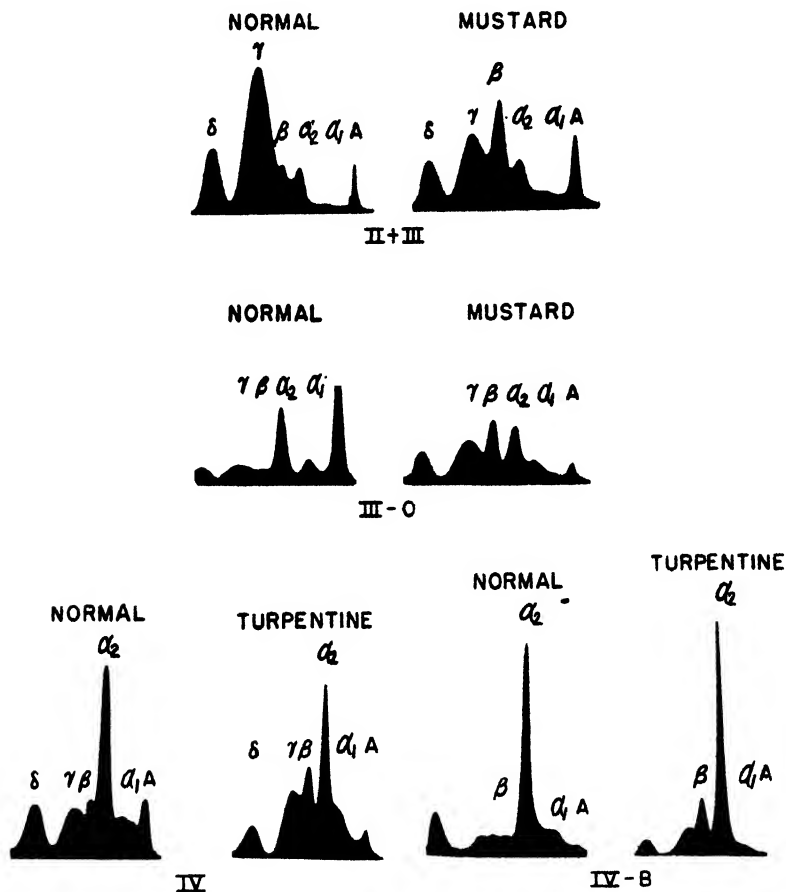


FIG. 3. Ascending electrophoretic patterns of fractions of sera from control and injured goats.

percentage distribution of various protein components is affected slightly after injury. The percentage of  $\beta$ -globulin increases in fractions II+III and III-0 of turpentine and H-gassed goats, but this is not observed in animals treated by cutaneous application of H. Increases are also seen in  $\beta$ -globulin in fractions IV and IV-B; these differences can be seen in the electrophoretic patterns (Fig. 3). Daily analyses for the percentage



distributions and concentrations of proteins of whole sera in individual gassed goats are presented in Table II. The albumin decreases immedi-

TABLE II  
*Distributions and Protein Concentrations of Serum Proteins of Goat Exposed to Ct 71,000\**

Days after treatment	Per cent distribution					Gm. protein per 100 ml. serum				
	Albumin	$\alpha_1$	$\alpha_2$	$\beta$	$\gamma$	Albumin	$\alpha_1$	$\alpha_2$	$\beta$	$\gamma$
0	54	5	15	4	21	3.7	0.3	1.0	0.3	1.4
1	42	10	13	6	28	2.5	0.6	0.8	0.4	1.7
2	37	13	14	10	26	2.1	0.7	0.8	0.6	1.5
3	42	14	12	15	17	3.1	1.0	0.9	1.1	1.2
5	39	14	12	14	19	2.9	1.0	0.9	1.0	1.4
6	40	13	11	15	20	2.5	0.8	0.7	0.9	1.3

\*The expression Ct represents the mg. of mustard (C) multiplied by the time in minutes (t) per cu.m.

TABLE III  
*Electrophoretic Analyses of Sera of Burned Goats*

Run No.	Days after burn	Per cent distribution*					Gm. N per 100 ml. serum				
		Albu- min	$\alpha_1$	$\alpha_2$	$\beta$	$\gamma$	Albu- min	$\alpha_1$	$\alpha_2$	$\beta$	$\gamma$
1	0	46	9	11	6	27	0.58	0.11	0.14	0.08	0.34
	1	44	12	14	6	24	0.46	0.13	0.15	0.06	0.25
	2	36	16	15	10	23	0.37	0.16	0.15	0.10	0.23
2	0	30	13	9	12	36	0.37	0.16	0.11	0.15	0.44
	1	26	13	13	14	34	0.26	0.13	0.13	0.14	0.34
	2	23	13	11	26	27	0.27	0.15	0.13	0.30	0.32
3	0	46	9	13	6	26	0.51	0.10	0.14	0.07	0.29
	1	43	11	15	7	23	0.43	0.11	0.15	0.07	0.23
	2	38	16	16	9	21	0.36	0.15	0.15	0.09	0.20
4	3	34	19	15	11	20	0.32	0.18	0.14	0.10	0.19
	0	39	12	13	8	28	0.44	0.14	0.15	0.09	0.32
	1	39	13	14	7	26	0.38	0.13	0.14	0.07	0.25
	2	35	17	14	9	25	0.34	0.16	0.14	0.09	0.24
	3	33	17	16	14	20	0.32	0.17	0.16	0.10	0.20

\* Ascending boundaries.

ately and remains low; the  $\alpha_1$ - and  $\beta$ -globulins increase and remain elevated during the period of observation.

The yields of fractions in terms of dry weight and in nitrogen for a goat injured with turpentine indicate that this injury is responsible for an absolute increase in the amount of fraction II+III and a decrease in albumin. Subfraction III-0, which contains most of the  $\beta$ -globulin of II+III, increased above the corresponding values for the control animal.

**Thermal Injury**—Electrophoretic analyses of the whole sera of severely burned goats show that the albumin and  $\gamma$ -globulin decrease, while the  $\alpha_1$ - and  $\beta$ -globulins increase slightly (Table III). Nitrogen determinations indicate that the absolute amounts of albumin and  $\gamma$ -globulin decrease in each of four burned goats. The  $\beta$ -globulin concentrations increased in

TABLE IV  
*Lipide Analyses in Serum Protein Fractions of Control Goat*

Fraction	Total cholesterol C	Free cholesterol C	Phospholipide C	Total lipid C	Carbon of lipid constituents accounted for*
	gm. per cent	gm. per cent	gm. per cent	gm. per cent	per cent
Serum	0.78	0.13	0.60	2.34	76
II+III	0.86	0.23	0.43	2.20	79
IV	0.94	0.11	0.97	3.05	78
IV-4†	1.52	0.18	1.34	4.35	85
V	0.55	0.06	0.57	1.70	84
II-1,2	0	0	0	0.45	0
II-3	0	0	0	0.69	0
III-2	2.47	0.78	1.41	7.35	67
III-3	0.64	0.21	0.53	3.00	48
IV-W, 1	1.29	0.16	1.00	2.88	104
IV-A	2.62	0.29	2.37	7.70	83
IV-B	0.32	0.04	0.06	0.48	103
IV-4A	0.94	0.10	0.50	2.07	98
IV-4B	0.72	0.08	0.39	1.38	108
IV-4C	0.66	0.10	0.89	2.32	81
V-1	2.33	0.38	1.87	5.86	92
V-2	0.23	0.03	0.60	2.58	38
V-3	0	0	0	0.55	14

\* Cholesterol + phospholipide + ester cholesterol fatty acid carbon (gm. per cent).

† Includes IV-4, 1.

only one goat and the concentration of  $\alpha_1$ -globulin nitrogen increased slightly in three of the four experiments.

Sera obtained from goats 12, 24, 48, and 72 hours after burning were fractionated. The percentage distribution of the proteins determined from the electrophoretic pattern showed no outstanding change.

**Lipide Analyses**—The data for total lipid C, total and free cholesterol, and phospholipide for the serum, main fractions, and subfractions of control and injured goats are shown in Tables IV and V. The concentra-

tions of the lipid constituents of goat serum fractions are low compared to those observed for similar fractions of dog serum.

In the control serum (Table IV), fractions III-2 and IV-A have the highest total lipid C concentrations of 7.4 and 7.7 per cent respectively. The lowest values are observed for the two electrophoretically pure  $\gamma$ -globulins, II-1, 2 and II-3, and the  $\alpha_2$ -globulin-rich fraction IV-B and pure

TABLE V

*Lipide Analyses of Sera and Fractions of Goat at Varying Intervals after Burning*

Fraction	After burn	Total cholesterol C	Free cholesterol C	Phospholipide C	Total lipid C	Carbon of lipid constituents accounted for*
	<i>hrs.</i>	<i>gm. per cent</i>	<i>gm. per cent</i>	<i>gm. per cent</i>	<i>gm. per cent</i>	<i>per cent</i>
Serum	12	0.64	0.13	0.02	1.56	74
II+III		0.97	0.21	0.04	1.39	106
IV		1.35	0.18	0.82	3.15	91
IV-4†		0.75	0.09	0.34	1.72	87
V		0.14	0.03	0.05	0.38	68
Serum	24	0.79	0.15	0.24	2.07	69
II+III		0.93		0.24	1.33	
IV		3.00		1.45	4.54	
IV-4		0.89		2.13	2.72	
V		0.03		0.20	0.92	
Serum	48	0.69		0.30	2.24	
II+III		1.15	0.31	0.10	1.70	104
IV		1.09	0.22	0.59	2.87	76
IV-4		1.48	0.28	1.21	3.80	80
V		0.14	0.03	0.12	1.28	24
Serum	72	0.80	0.14	0.37	2.69	59
II+III		1.36		0.26	2.22	
IV		2.26	0.32	1.75	5.34	97
IV-4		0.88	0.13	0.67	2.70	75
V		0.04		0.02	0.42	

\* Fractions IV-4 and V obtained by modified procedure.

† Includes IV-4, 1.

albumin V-3. The percentage of free cholesterol is greatest in fraction II+III and its subfractions, and the variations in concentration are roughly proportional to those of the total lipid C. The two pure  $\gamma$ -globulins and albumin contain no cholesterol. The phospholipide concentrations of the different fractions show large variations. The total lipid carbon can be accounted for as cholesterol and phospholipide in three of the fourteen fractions shown in Table IV. It is assumed that lipid unaccounted for is present as fatty acids or neutral fat.

The lipid analyses of fractions obtained from the serum of goats treated

by cutaneous application of H indicate that no outstanding changes occur in lipid distribution.

Data for the lipid analyses of the main serum fractions of goats sacrificed at intervals of 12, 24, 48, and 72 hours after being burned are shown in Table V. The percentage concentration of total cholesterol of whole serum remains fairly constant. After the animal has been burned, the cholesterol content of fraction V decreases and the substance is present only in traces.

The phospholipide concentrations of the whole sera are consistently lowered. The phospholipide values for all fractions are markedly decreased 12 hours after injury; the values for fractions II+III and V remained depressed during the period of observation; results obtained for fractions IV and IV-4 are variable. The total lipid carbon concentrations of the whole sera remain fairly constant but differ considerably in the various fractions. Constant decreases are noted for fractions IV-4 and V after injury.

*Hexosamine*—The hexosamine concentrations of many of the fractions of control and injured animals were determined but are not listed. The lowest value for a main fraction is 0.72 per cent for fraction V; the pure albumin (V-3) has a value of 0.16 per cent. The highest value of 2.9 per cent is obtained for fraction IV-4A, which is composed chiefly of  $\alpha_1$ -globulin. The remaining values vary between 1.1 and 2.0 per cent. A value of 2.8 per cent was obtained for a  $\gamma$ -globulin-rich fraction (IV-4) in dog serum.

#### DISCUSSION

Severe injury in the goat causes relatively small changes in the distribution of the protein components of whole serum or its fractions and in lipid concentrations. This is in contrast to the marked changes noted for the protein and lipid distributions and concentrations in the sera of injured dogs. These differences between the response of the goat and dog to injury may reflect the differences in the lipid metabolism in herbivorous and carnivorous species. It may be postulated that lipides may be associated with the synthesis of certain plasma proteins. From these experiments with the goat, it can be assumed that results obtained for the proteins of goat plasma cannot be applied to other species.

#### SUMMARY

New procedures are described for separating fractions IV, IV-4, and V and their subfractions from goat serum according to principles presented by Cohn and his associates. Modifications are necessary because of the unusual solubility of goat serum albumin.

The mobilities and percentage distribution of the main and subfractions of sera of control and injured (turpentine, bis( $\beta$ -chloroethyl)sulfide, and heat) goats are presented. Two  $\gamma$ -globulins and albumin can be obtained in electrophoretically pure form. In addition, fractions extremely rich in  $\alpha_1$ - and  $\alpha_2$ -globulins can be isolated. Analyses for total and free cholesterol, phospholipide and total lipide carbon, and hexosamine are presented for the sera and fractions of control and injured goats. Only small changes are seen in the concentrations of these lipide components after injury.

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# A CELLULOLYTIC ENZYME PREPARATION FROM MYROTHECIUM VERRUCARIA

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Cellulolytic enzyme preparations active against various forms of cellulose have been reported by a number of investigators who have used a variety of different sources (8). Fungi which have been employed for the preparation of such extracts are *Sporotrichum carnis* (15), *Aspergillus oryzae* (3, 2), *Merulius lachrymans* (9), and *Contiophora cerebella* (7), while bacteria have included *Bacterium protozoides* (5), *Bacterium bosporum* (5), *Cellulobacillus myxogenes* (14), *Cellulobacillus mucosus* (14), *Plectridium cellulolyticum* (10), several species of *Cytophaga* (1), and various thermophilic cellulose-decomposing bacteria (11). In addition, active cellulolytic extracts have been prepared from the intestinal juice of the snail *Helix pomatia* (6), from green malt (12), and from the protozoon *Eudiplodinium neglectum* which was isolated from cattle rumen (4). Although the data presented by several of these investigators are certainly suggestive of true cellulolytic enzyme activity, nevertheless these workers in general have not conclusively established the cell-free nature of their preparations. This is of considerable importance in view of failure of other investigators to obtain cell-free cellulolytic enzymatic preparation from fungi. Questions were raised as to the possibility of bacterial or fungal contamination during the period (usually several days or more) of testing for cellulolytic activity.

The present paper describes the preparation and some of the properties of a cell-free cellulolytic enzyme from *Myrothecium verrucaria*, which has been shown to be one of the most active of the cellulose-destroying fungi isolated from deteriorated fabrics (16).

## EXPERIMENTAL

*Preparation of Enzymatic Extract*—300 gm. of filter paper (E and D No. 615) were ground through a 40 mesh sieve in a Wiley mill and autoclaved in a 5 gallon Pyrex bottle, to which were added 10 liters of a sterile mineral solution of the composition shown in Table I. After cooling, the flask was inoculated with 100 ml. of a spore suspension of *Myrothecium verrucaria*, United States Department of Agriculture No. 1334.2, containing about

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80,000 spores per ml., from a 3 week culture of the fungus grown on filter paper. Sterile aeration was provided by passing the air through a water tower and a cotton filter at a rate of about 500 ml. per minute. At the same time, the bottle was shaken at a rate of about fifteen rotary cycles per minute in order to keep the ground filter paper in suspension. The entire set-up was kept in a room maintained at 30°. After 10 days incubation the cellulose-mycelium mixture was filtered on a Büchner funnel, and the yellow filtrate containing the bulk of the cellulolytic activity was stored at 5° in the presence of toluene as an antiseptic. No appreciable loss of activity was observed over a period of several months.

### *Testing Method for Cellulolytic Activity*

An index of cellulolytic activity of the different preparations was obtained by the amount of reducing substances formed from cellulose under

TABLE I  
*Composition of Mineral Solution*

	gm.		mg.
$\text{KH}_2\text{PO}_4$	0.20	$\text{Fe}_2(\text{SO}_4)_3 \cdot 6\text{H}_2\text{O}$	0.054
$\text{K}_2\text{HPO}_4$	0.15	$(\text{NH}_4)_2\text{P}(\text{MoO}_4\text{O}_{10})_4$	0.024
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	2.00	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.050
$\text{Na}_2\text{HPO}_4$	1.50	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.0025
$\text{NH}_4\text{NO}_3$	0.60	$\text{MnSO}_4$	0.0055
$\text{NaNO}_3$	3.80	$\text{H}_2\text{BO}_3$	0.057
			ml.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.30	$\text{H}_2\text{O}$	1000

standard conditions. In the test, 5 ml. of the dialyzed test solution were added to 5 ml. of a 0.05 M phosphate buffer (pH 5.0) and 100 mg. of de-waxed cotton, which had been pulverized in a ball mill for 24 hours. 5 drops of toluene were added as an antiseptic to the mixture in a 125 ml. Erlenmeyer flask. After incubation for 4 days at 30°, the mixture was filtered and the amount of reducing sugars in the filtrate was determined by titration (13).

In order to insure that the action of the cellulolytic enzymes and not of contaminating microorganisms is determined by the method, aliquot samples of the enzyme solution were tested with and without sterilization by Berkefeld filtration, as well as with and without the addition of fungal spores to the test flasks. The results given in Table II clearly show that the conversion of cellulose into reducing sugars is not brought about by contaminating organisms.

The percentage of cellulose breakdown, when cellophane and pulverized

filter paper and cotton were used as substrates, was also determined in several experiments by loss in weight of cellulose, and the values so obtained checked within 20 per cent with those obtained by titration, assuming glucose to be the product of the enzymatic hydrolysis.

### *Properties of Enzyme Preparation*

*Extracellular Nature*—Experiments were conducted to determine whether the cellulolytic enzyme (or enzymes) was intra- or extracellular in nature.

TABLE II  
*Cell-Free Nature of Cellulolytic Preparations*

Treatment of enzyme preparation	Spores added	Toluene added	Cellulose breakdown*
		ml.	per cent
Untreated	0	1.0	11.8
	+	1.0	12.8
Berkefeld filter	0	0	9.0
	0	1.0	10.5
Control		0	0
		1.0	0

\* Calculated from the reducing sugar formed, assuming glucose to be the only sugar present.

TABLE III  
*Cellulolytic Activity of Metabolic Filtrate and Mycelial Extract of *Myrothecium verrucaria**

Substrate	Per cent cellulose breakdown		
	Metabolic filtrate (a)	Mycelial extract (b)	(a) + (b)
Ground cellophane . . . . .	28.0	6.0	23.0
Pulverized filter paper . . . . .	8.0	3.0	9.0
Xylan . . . . .	14.5	6.0	

*Myrothecium verrucaria* was cultured for 5 days on ground filter paper in the manner described above. The metabolic mixture was filtered, giving a clear yellowish filtrate and a residue of mycelium and undigested cellulose. The residue was ground with sand in a volume of culture medium equivalent to the filtrate originally present. A third test solution was made through a combination of the first two. The three solutions were dialyzed against distilled water for 24 hours in cellophane tubing and tested for cellulolytic activity with different substrates. Results of the 5 day test are given in Table III. The figures obtained strongly suggest that the



cellulolytic activity is present in the filtrate and not in the mycelium. The low activity in the so called "mycelial extract" may be due to the filtrate absorbed by the residue of mycelium plus undigested cellulose.

*Temperature Stability and Optimum*—In testing for heat stability, aliquot portions of the same preparation at pH 6.0 were kept for 10 minutes at different temperatures and then tested for activity. Results are given in Table IV, which indicate that 50° is the threshold value at which inactivation begins.

TABLE IV  
*Heat Stability of Cellulolytic Preparation*

Temperature (10 min. standing)	Cellulose breakdown
°C.	per cent
22	13.1
30	13.2
40	13.5
50	8.4
60	2.7
70	0.0

TABLE V  
*Temperature Optimum of Cellulolytic Preparation*

Incubating temperature	Cellulose breakdown
°C.	per cent
0	4.7
3	6.2
25	13.9
31	13.9
40	16.0
50	4.2

The optimum temperature for enzymatic action was studied by incubating the enzyme-buffer-pulverized filter paper mixture for 4 days and determining the percentage conversion of cellulose into reducing sugars. The results show an optimum around 40° (Table V).

*pH Stability and Optimum*—The stability of the crude enzymatic solution was also studied. Different solutions were adjusted to their respective pH values with dilute hydrochloric acid and sodium hydroxide, allowed to stand for 48 hours, readjusted to pH 5.0, then assayed for cellulolytic activity in the usual manner. The results of the experiment, given in Table VI, show rapid inactivation beyond the limits of pH 5.0 and 9.0.

A series of 0.05 M phosphate buffers of pH 4.0, 5.0, 6.0, and 7.0 was prepared to determine the pH at which the greatest rate of cellulolytic action takes place. Various enzymatic aliquot solutions were adjusted with phosphoric acid and sodium hydroxide to the same respective values. The cellulolytic activity at these various pH values was tested against the pulverized dewaxed cotton in the usual way. The results in Table VII indicate a pH optimum of 5.5.

TABLE VI  
*Stability of Enzymatic Preparation at Different pH Values*

pH (48 hrs. standing)	Cellulose breakdown
	<i>per cent</i>
1.0	0.0
3.0	2.7
5.0	8.5
7.0	11.6
9.0	8.4
11.0	0.0
13.0	0.0

TABLE VII  
*Optimum pH of Cellulolytic Enzymes*

pH	Cellulose breakdown
	<i>per cent</i>
4.0	8.5
5.0	14.3
6.0	14.2
7.0	6.9

*Concentration Curve*—Dialyzed metabolic filtrate from *Myrothecium verrucaria* grown on ground filter paper was concentrated at 20 mm. mercury vacuum at a bath temperature of 40–50° for 6 hours to one-fiftieth of its original volume. Different dilutions of this concentrate were tested for their cellulolytic rates against pulverized filter paper. The results are plotted in Fig. 1.

*Product of Enzymatic Breakdown*—250 ml. of the enzymatic preparation and 250 ml. of 0.05 M phosphate buffer, pH 5.0, were added to 5.0 gm. of pulverized dewaxed cotton. After the addition of 10 ml. of toluene as an antiseptic the stoppered Erlenmeyer flask was incubated for 14 days at 30° with daily gentle shaking. Shaffer-Somogyi titration of the metabolic filtrate at the end of the incubation period showed a conversion of 22.9 per

cent of the cellulose into reducing sugars, calculated as glucose. 100 ml. of the filtrate, containing 250 mg. of glucose according to the titration, were concentrated *in vacuo* to 7 ml. The osazone was prepared with a yield of 215 mg., and after two recrystallizations from 50 per cent ethanol, melted at 201–203°. A simultaneously prepared glucosazone showed a melting point of 200–202° with a mixed melting point of 197–201°. Cello-biosazone gave a value of 196–198° and a mixed melting point of 180–185°. These data clearly indicate that glucose is one of the main products from the enzymatic breakdown of cellulose.

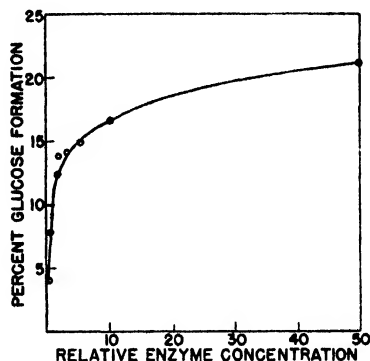


FIG. 1. Effect of enzyme concentration on rate of cellulolytic action

#### SUMMARY

A method has been described for the preparation of a cell-free enzymatic preparation from the fungus, *Myrothecium verrucaria*, capable of degrading cellulose. The cellulolytic enzyme (or enzymes) was extracellular, with an optimum temperature of 40° and an optimum pH of 5.5 when acting on cotton. It is rapidly inactivated at temperatures above 50° and at values beyond the limits of pH 5.0 and 9.0. The main breakdown product from cellulose was shown to be glucose.

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<sup>1</sup> All melting points were taken in capillary tubes and are uncorrected.

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# THE UTILIZATION OF PROLINE DERIVATIVES BY MUTANT STRAINS OF *ESCHERICHIA COLI*\*

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It has been reported recently (1, 2) that *phenylalanineless*, *tyrosineless*, and *leucineless* mutant strains of *Escherichia coli* may utilize, for growth, peptides of the appropriate amino acid. These peptides do not exhibit any greater growth-promoting action, however, than is shown by the parent amino acids. It was suggested, therefore, that, prior to utilization for growth, the peptides are hydrolyzed by the bacterial enzymes, thus liberating the amino acid required as a growth factor. In the present communication there are reported the results of a study of the utilization of peptides by two *prolineless* mutant strains of *Escherichia coli*. Of especial interest is the finding that, for one of these two strains, proline peptides are much more effective growth factors than is proline itself.

The origin of the two proline-requiring mutants (strains 679-183 and 679-662) has been described by Tatum (3). Both strains are double mutants of *Escherichia coli* strain K-12, and require threonine as well as proline. The present discussion will be limited to the proline requirement, and all tests were made in the presence of ample DL-threonine (0.5 mg. per 10 ml. of medium) to support optimal growth.

As reported by Tatum (3), the mutants exhibit different requirements for proline; strain 679-183 grows only in the presence of proline, while strain 679-662 can use glutamic acid in place of proline. Neither strain can use ornithine, arginine, or hydroxyproline in lieu of proline (3). For convenience, strain 679-183 will be referred to here as strain I, the proline-requiring mutant, and strain 679-662 will be termed strain II, the proline-glutamic acid-requiring mutant. The availability of two such mutants has made it possible to compare the utilization of the same peptide by strains which differ in their growth requirements and, also, to compare the efficacy of peptides of proline or glutamic acid as growth factors for a single strain which grows in the presence of either amino acid.

The rate of growth of the two strains in a complete synthetic medium is quite different; in the presence of proline, the growth of strain I is considerably more rapid than that of strain II in the presence of proline or of glu-

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tamic acid. Essentially complete growth of strain I is obtained within 24 hours at 30° or within 48 hours at 25°, while with strain II 64 hours are required for complete growth at 25°.

Another difference between the two mutants lies in the stability of the mutations responsible for the proline requirement. Strain I is quite stable and may be cultured for 3 days at 30° without any detectable change in its proline requirement. Strain II, however, is relatively unstable and often loses its proline-glutamic acid requirement in 48 hours at 30° or in 72 hours at 25°. While the strain apparently regains the ability to synthesize proline and glutamic acid, it does not show any change in its requirement for threonine.

Since it was desirable to compare the activity of proline peptides as growth factors for strains I and II, growth tests with both strains were conducted in colorimeter tubes incubated at 25° for a total of 64 hours, and the extent of growth was measured at 48 and 64 hours by density determination with the Evelyn photoelectric colorimeter. Even under these conditions, however, strain II occasionally reverted to the prototroph. Therefore, at the same time that the density was measured, routine tests for such reversion were made by sterile transfer of a loopful of the bacterial suspension from the colorimeter tube to 10 ml. of minimal medium (containing threonine). Detectable growth in the minimal medium after 24 to 36 hours incubation at 25° was taken as evidence for the loss of the mutation. When this occurred, the compound was retested until reliable data were obtained.

In all other respects, the methods employed for the study of the growth-promoting activity and of the possible sparing action of test compounds, as well as for the determination of the quantity of active compounds required to produce half maximal growth of the mutants, were similar to those described previously for the *leucineless* mutant of *Escherichia coli* (2). As before, the relative activity of a peptide or amino acid derivative as compared to that of the parent amino acid was calculated from growth curves obtained at the same time.

Under the specified experimental conditions, the quantity of L-proline required by strain I for half maximal growth in 48 hours is 0.24 micromole per 10 ml. of medium and, at 64 hours, it is 0.23 micromole. Strain II required, for half maximal growth at 48 hours, 0.56 micromole of L-proline or 1.9 micromoles of L-glutamic acid. At 64 hours the requirement of strain II is 0.44 micromoles of L-proline or 0.44 micromole of L-glutamic acid. It may be seen from these values that, although the efficacy of proline and glutamic acid as growth factors for strain II is the same at the end of 64 hours, the response to glutamic acid is very much less than is the response to proline when the two are compared at 48 hours.

It has been noted previously (4, 5) that some proline peptides readily undergo cyclization to form diketopiperazines. In testing such peptides for their utilization by the mutant strains of *Escherichia coli*, it was essential that solutions of the test substances be sterilized by filtration through a bacterial filter rather than by autoclaving.

### *Response of Strain I to Proline Derivatives*

*Proline Peptides*—As shown in Table I, all of the di- and tripeptides tested with strain I under the conditions outlined above were found to be

TABLE I

### *Response of Strain I to Proline Peptides*

Unless otherwise noted, all proline peptides were tested by adding filter-sterilized solutions of the test compound to autoclaved media.

Compound*	Quantity, per 10 ml., for half maximal growth	
	48 hrs.	64 hrs.
	micromoles	micromoles
L-Proline (6).....	0.24	0.23
L-Prolylglycine (4, 7).....	0.12	0.09
L-Proline + glycine.....	0.24 of each	0.23 of each
L-Prolyl-L-leucine (4, 7).....	0.18	0.17
L-Prolyl-L-glutamic acid†.....	0.10	0.08
L-Proline + L-glutamic acid.....	0.24 of each	0.25 of each
L-Prolyl-L-tyrosine (4, 7).....	0.12	0.09
Glycyl-L-proline (8).....	0.14	0.115
"        ‡.....	0.34	0.39
Glycyl-L-proline diketopiperazine (5)‡.....	24 (Ca.)	17.3
Glycyl-L-prolylglycine†.....	0.075	0.07
Diglycyl-L-proline (5).....	0.10	0.06

\* The figures in parentheses refer to bibliographic reference numbers.

† The synthesis of this compound is described in the text.

‡ The solution of this substance was sterilized by autoclaving.

more active than proline. This increased activity is observed at both the 48 and 64 hour readings, as is noted in Table I, and is detectable within 24 hours as well, although complete growth is not attained at this time. It may be added that the relative activity of L-prolyl-L-glutamic acid is exactly the same when the tests are conducted at 30° as that noted at 25°.

The effect of autoclaving on the activity of proline peptides may be seen from the data given in Table I for glycyl-L-proline. Although glycyl-L-proline diketopiperazine is an active growth factor for strain I, it is about 150 times less active than is a filter-sterilized solution of the free dipeptide. When the latter compound is autoclaved, it loses much of its activity and



this loss may be ascribed to the formation of the diketopiperazine during heating in the autoclave. It would appear that the diketopiperazine ring must be opened by the mutant before the cyclic compound can be used for growth.

The high relative activity of all of the proline peptides for strain I cannot be due merely to the fact that they provide a source of amino nitrogen, or an amino acid other than proline. The data in Table I show that, when proline is supplemented with equimolar amounts of glycine or of L-glutamic acid, the effect on growth is no greater than that caused by L-proline alone. A reasonable explanation of the data seems to be that, in the case of strain I, the proline peptides can be utilized as such and may be incorporated into cellular proteins without prior hydrolysis to yield proline.

The activity of proline peptides for strain I is not a general phenomenon for the biochemical mutants of *Escherichia coli* strain K-12. As will be shown in a later section of this paper, strain II does not exhibit the same growth response to proline peptides as that found for strain I. Furthermore, tests with a *tyrosineless* strain (1) showed that L-prolyl-L-tyrosine is no more active for this strain than is L-tyrosine, and, in the case of a *leucineless* mutant (2), L-prolyl-L-leucine is no more active than is L-leucine. However, when the *prolineless* strain 58-6317 (9), which requires proline and biotin but not threonine, was tested with L-proline, L-prolylglycine, L-prolyl-L-tyrosine, L-prolyl-L-glutamic acid, and L-proline supplemented with L-glutamic acid, the results were identical with those obtained with strain I. This may be taken as evidence to show that, although the *Escherichia coli* strains I and 58-6317 differ with respect to their requirements for biotin and threonine, the mutation responsible for the proline requirement is the same in the two mutants.

*Other Proline Derivatives*—In addition to the peptides listed above, a number of other compounds containing proline were tested for their effect on the growth of strain I. It will be noted from Table II that the relative activities of prolinamide, acetylproline, carbobenzoxyglycylproline, and carbobenzoxyprolylproline are similar to the data obtained for analogous derivatives of tyrosine, phenylalanine, and leucine when these were tested with the appropriate mutants (1, 2). It is of interest that prolinamide appears to undergo transformation when it is autoclaved in the minimal medium. The relatively high activity of such autoclaved preparations is in sharp contrast to the low activity of filter-sterilized solutions of this substance. It may be added that when prolinamide hydrochloride is autoclaved in water and the resultant solution is added to the autoclaved medium, it exhibits the same growth-promoting activity as does the filter-sterilized material.

The behavior of acetyldehydrophenylalanyl-L-proline is also of special

TABLE II

*Response of Strain I to Derivatives of Proline*

Unless otherwise noted, the compounds were dissolved in the medium and sterilized by autoclaving.

In this table, — indicates that the compound was inactive at the specified time; + indicates that the compound was active but did not give half maximal growth at the highest concentration used in the test.

Compound*	Quantity, per 10 ml., for half maximal growth		Maximal quantity, per 10 ml., used in test†	Remarks
	48 hrs.	64 hrs.		
	micro-moles	micro-moles	micromoles	
L-Proline	0.24	0.23		
L-Prolinamide·HCl (5)	9.8	6.7		
“ ‡	—	+	19.9 [0.1]	No sparing action
Acetyl-L-proline (10)	—	—	19.1	
Carbobenzoxy-L-prolyl-L-proline (4)	+	+	14.5 [0.07]	
Carbobenzoxyglycyl-L-proline (8)	+	+	26.1 [0.18]	
Acetyldehydrophenylalanyl-L-proline (11)	4.3	4.1		
Acetyldehydrophenylalanyl-L-proline‡	±	+	16.5 [0.05]	
Acetyl-L-phenylalanyl-L-proline (11)	±	+	9.9 [0.06]	
Dehydrophenylalanyl-L-proline diketopiperazine (12)	—	—	17.6	No sparing action
Hydroxy-L-proline (6)	—	—	38.1	“ “ “
Glycylhydroxy-L-proline (9)‡	—	—	26.6	“ “ “
L-Arginine·HCl	—	—	14.3	“ “ “
L-Ornithine·2HCl	—	—	14.6	“ “ “
L-Glutamic acid	—	—	6.8	“ “ “
L-Glutamine (13)‡	—	—	6.9	“ “ “
L-Glutamyl-L-glutamic acid (14)	—	—	3.6	“ “ “
Glycyl-L-glutamic acid (15)	—	—	16.1	“ “ “

\* The figures in parentheses refer to bibliographic reference numbers.

† The figures in brackets denote the amount of proline, in micromoles per 10 ml., which produces the growth obtained with the maximal quantity of test substance employed.

‡ A filter-sterilized solution of this substance was added to the autoclaved medium.

interest. When a filter-sterilized solution of this compound is used, the growth of strain I is extremely slow. However, when the compound is dissolved in the minimal medium and sterilized by autoclaving, it exhibits

a marked growth-promoting action. On the other hand, similarly autoclaved solutions of acetyl-L-phenylalanyl-L-proline have very slight activity and dehydrophenylalanyl-L-proline diketopiperazine is inactive. It appears likely that, in the process of autoclaving, acetyldehydrophenylalanylproline is hydrolyzed (16) to yield the corresponding ketoacylamino acid, phenylpyruvyl-L-proline, which might then be responsible for the observed activity. If this were so, strain I could either hydrolyze phenylpyruvylproline to give proline or aminate it to form phenylalanylproline. In this connection it may be noted that previous work (1) has shown that the *phenylalanineless* mutant could not utilize phenylpyruvylglycine in place of phenylalanine, and this observation was taken to indicate that this strain was unable either to hydrolyze or to aminate the ketoacylamino acid. An autoclaved solution of acetyldehydrophenylalanylproline, which was active for strain I, was inactive for the *phenylalanineless* strain.

The other compounds listed in Table I showed no detectable effect on the growth of strain I under the experimental conditions employed.

#### *Response of Strain II to Derivatives of Proline and Glutamic Acid*

As is shown in Table III, although the addition of L-proline to the minimal medium produces a much faster initial growth of strain II than does the addition of L-glutamic acid, the activity of these two amino acids is exactly the same on a molar basis when growth is complete at 64 hours. An equimolar mixture of proline and glutamic acid does not give as rapid growth as does the equivalent amount of proline; 0.36 micromole of L-proline plus 0.36 micromole of L-glutamic acid is needed to produce half maximal growth in 48 hours while only 0.56 micromole of L-proline is needed to produce the same effect. The mixture, however, gives much more rapid growth than an equivalent amount of L-glutamic acid; the quantity of L-glutamic acid required for half maximal growth in 48 hours is about 1.9 micromoles. At 64 hours, the mixture has approximately the same activity as either of the amino acids alone.

When proline and glutamic acid are supplied as the dipeptide L-prolyl-L-glutamic acid, the initial growth rate is very slow, and half maximal growth is not attained in 48 hours, even in the presence of 12.3 micromoles of the dipeptide. Rapid growth occurs in the period between 48 and 64 hours, and at the time of the final reading the dipeptide is almost as active as a mixture of the constituent amino acids. It would appear, therefore, that the dipeptide is hydrolyzed before the proline and glutamic acid can be utilized for the growth of strain II.

*Other Proline Peptides*—Unlike strain I, strain II utilizes peptides other than prolylglutamic acid very poorly. The compounds tested showed little or no activity in 48 hours and glycyl-L-proline and diglycyl-L-proline,

TABLE III

*Response of Strain II to Derivatives of Proline and Glutamic Acid*

Unless otherwise noted, the compounds were dissolved in the medium and sterilized by autoclaving.

In this table, — indicates that the compound was inactive at the specified time; + indicates that the compound was active but did not give half maximal growth at the highest concentration used in the test.

Compound*	Quantity, per 10 ml., for half maximal growth		Maximal quantity, per 10 ml., used in test	Remarks
	48 hrs.	64 hrs.		
	<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>	
L-Proline	0.56	0.44		
L-Glutamic acid	1.9 (Ca.)	0.44		
L-Proline + L-glutamic acid	0.36 of each	0.25 of each		
L-Prolyl-L-glutamic acid†	+	0.31	12.3	
L-Prolinamide·HCl†	—	—	20.0	No sparing action
L-Prolyl-glycine†	—	+	17.4 [0.09]‡	
L-Prolyl-L-leucine†	±	22 (Ca.)	22.0	
L-Prolyl-L-tyrosine†	—	+	10.8 [0.2]‡	
Glycyl-L-proline†	±	10 (Ca.)	17.4	
Glycyl-L-proline diketopiperazine	—	—	19.5	No sparing action
Diglycyl-L-proline†	+	9 (Ca.)	19.4	
L-Glutamine†	0.76	0.57		
L-Isoglutamine (14)	±	20.5 (Ca.)	20.5	
L-Glutamyl-L-glutamic acid	0.95	0.35		
L-Glutamyl-L-phenylalanine (1)	±	0.70	10.3	
L-Glutamyl-L-tyrosine (17)	+	0.75	10.3	
Glycyl-L-glutamic acid	9.5	0.57		
Glycyl-L-glutamic acid diketopiperazine (18)	—	+	26.9 [0.08]§	
Glycyl-L-glutamyl-L-tyrosine (19)	—	+	8.2 [0.2]§	
Acetyl-L-glutamic acid (20)	—	—	26.5	No sparing action
L-Arginine·HCl	—	—	14.3	
L-Ornithine·2HCl	—	—	14.7	“ “ “

\* The figures in parentheses refer to bibliographic reference numbers.

† A filter-sterilized solution of this substance was added to the autoclaved medium.

The figures in brackets denote the amount of proline (‡) and the amount of glutamic acid (§), in micromoles per 10 ml., which produce the growth obtained with the maximal quantity of test substance employed.

which exhibit the highest activity, are only about 5 per cent as active as L-proline at the time of the final reading.

The response of strain II to proline peptides is unexpected in view of earlier experiments which showed that peptides have growth-promoting properties which compare favorably with those of the parent amino acids for the *phenylalanineless*, *tyrosineless*, and *leucineless* mutant strains of *Escherichia coli*. As will be shown below, strain II is able to use peptides of glutamic acid without difficulty and it is probable, therefore, that the ready utilization of L-prolyl-L-glutamic acid by this strain should be ascribed to the presence of a glutamic acid residue in the peptide. The slight response of strain II to other proline peptides suggests that the mutation responsible for the proline-glutamic acid requirement may have altered in some way the ability of *Escherichia coli* to utilize peptides of proline.

*Glutamic Acid Derivatives*—The data in Table III show that L-glutamine is superior to L-glutamic acid as a growth factor when the two are compared at 48 hours, but is slightly less active than the free amino acid at 64 hours. On the other hand, L-isoglutamine exhibits only slight activity, suggesting that the hydrolysis of the  $\alpha$ -amide linkage is a prerequisite for the utilization of this substance. The low activity of isoglutamine is similar to that of the amides of the amino acids required by the mutants studied previously (1, 2).

L-Glutamyl-L-glutamic acid proved to be the most active of the glutamic acid peptides investigated. On an equimolar basis, it is twice as active as L-glutamic acid at 48 hours but, at 64 hours, it shows only about 80 per cent of the activity of glutamic acid. The latter relationship was also found to exist between L-tyrosyl-L-tyrosine and L-tyrosine when these were tested with the *tyrosineless* mutant (1).

The difficulty with which strain II utilizes proline peptides is brought out by a comparison of the activity of L-glutamyl-L-glutamic acid and of L-prolyl-L-glutamic acid. The mutant gives half maximal growth in 48 hours with 0.95 micromole of glutamylglutamic acid but half maximal growth does not occur in the presence of as much as 12.3 micromoles of prolylglutamic acid. If one assumes that both dipeptides must be hydrolyzed by the mutant prior to utilization for growth, and in view of the greater growth-promoting activity of proline as compared with glutamic acid, the presence of a proline residue in a peptide clearly reduces the rate at which strain II can hydrolyze the peptide.

The other peptides of glutamic acid which were tested, with the exception of glycyl-L-glutamic acid, showed little growth-promoting action during the first 48 hours. At 64 hours, however, all showed some activity, and the final values were similar to those found for the phenylalanine,

tyrosine, and leucine series in that the dipeptides are very active and the longer peptides less active. The data in Table III thus offer further evidence for the view that, in general, peptides are hydrolyzed by the bacterial enzymes before the constituent amino acids are utilized as growth factors. On the other hand, the response of strain I to proline peptides suggests that such hydrolysis may not be an obligatory step. The activity of proline peptides for strain I may be compared to that of strepogenin for certain hemolytic streptococci and for *Lactobacillus casei* (21-23).

The relationship between glutamic acid and proline which is seen for strain II has already been established for the rat (24, 25) and for *Penicillium notatum* (26). Since strain II behaves like analogous mutant strains of *Penicillium* (26) and does not grow on ornithine (Table III), which is a postulated intermediate in the conversion of proline to glutamic acid in the rat (27), the apparent formation of glutamic acid from proline in *Escherichia coli* appears to resemble that suggested for *Penicillium*. However, *Escherichia coli* seems to differ somewhat from *Penicillium* with respect to the proline-glutamic acid relationship, since ornithine exerts a sparing action on the utilization of glutamic acid by appropriate mutant strains of *Penicillium* but not by strain II.

#### Synthesis of Test Compounds

With the exception of two proline peptides, all the derivatives of proline or of glutamic acid used as test substances have been described previously.

**L-Prolyl-L-glutamic Acid**—To a solution of 3 gm. of L-glutamic acid in 20 ml. of 2 N NaOH, there were added, in small portions, an ethereal solution of carbobenzoxy-L-prolyl chloride (prepared from 2.3 gm. of L-proline by the method described in (4)) and 10 ml. of 2 N NaOH. The reaction mixture was shaken and chilled. At the end of the reaction, the solution was acidified with concentrated hydrochloric acid to yield a sirup which crystallized readily. This product (carbobenzoxy-L-prolyl-L-glutamic acid) was washed carefully with water and dried over phosphorus pentoxide (yield 3.2 gm.). 0.8 gm. of the carbobenzoxy compound was subjected to catalytic hydrogenation in the presence of palladium black. After carbon dioxide evolution had ceased, the catalyst was removed by filtration and the filtrate was evaporated to dryness. The resulting peptide was recrystallized from alcohol-water and dried at 100° *in vacuo* over phosphorus pentoxide.

$C_{24}H_{30}O_8N_2$	Calculated.	C 49.2, H 6.6, N 11.5
244.2	Found.	" 49.1, " 6.7, " 11.5
$[\alpha]_D^{25} = -49.0^\circ$ (2% in water)		

**Glycyl-L-prolylglycine**—To a solution of 0.86 gm. of L-prolylglycine (4, 7) in 5 ml. of N NaOH, there were added, in several portions, 1.4 gm. of carbo-

benzoxylglycyl chloride and 5.2 ml. of *N* NaOH. The reaction mixture was shaken and chilled. At the end of the reaction, the solution was acidified with concentrated hydrochloric acid to yield a sirup which was extracted with ethyl acetate. The ethyl acetate layer was dried over  $\text{Na}_2\text{SO}_4$  and evaporated *in vacuo* to give crystalline carbobenzoxylglycyl-L-prolylglycine. Yield 0.95 gm., m.p. 136–137°. This material contained 11.4 per cent N (theory for  $\text{C}_{17}\text{H}_{21}\text{O}_6\text{N}_3$ , 11.6). On hydrogenation of 0.45 gm. of the carbobenzoxyltripeptide in the usual manner, there was obtained 0.26 gm. of the free peptide. When dried in air, the substance forms a dihydrate. For analysis, it was dried at 100° *in vacuo* over phosphorus pentoxide.

$\text{C}_{17}\text{H}_{21}\text{O}_6\text{N}_3$ .	Calculated.	C 47.2, H 6.6, N 18.3
229.2	Found.	" 47.2, " 6.6, " 18.2
	$[\alpha]_D^{25}$	= -109.2° (2% in water)

The authors are indebted to Dr. E. L. Tatum for valuable advice in the course of this investigation.

#### SUMMARY

A mutant strain of *Escherichia coli* which requires an exogenous source of proline (strain I) and another mutant strain which requires an exogenous source of proline or of glutamic acid (strain II) have been studied with respect to their ability to utilize derivatives of these amino acids. Strain I was found to utilize peptides of proline much more effectively than proline. This is the first instance of a biochemical mutant strain of *Escherichia coli* which grows better in the presence of peptides of an essential amino acid than in the presence of the free amino acid. Although strain II grows more rapidly in the presence of proline than with glutamic acid, it utilizes proline peptides much less readily than glutamic acid peptides, and neither proline peptides nor glutamic acid peptides are more active as growth factors than the parent amino acids.

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# UTILIZATION OF SULFUR-CONTAINING AMINO ACIDS BY MUTANT STRAINS OF *ESCHERICHIA COLI*\*

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The production (1) of two double mutants of *Escherichia coli* which require for growth an exogenous source of a sulfur-containing amino acid and biotin has been described recently by Tatum (2). One strain, No. 58-161, was classified as a *methionineless* mutant, and the other, No. 58-309, as a *cystineless* mutant.

In the present communication, there are presented the results of a further investigation of the utilization of sulfur-containing amino acids by these two mutant strains. The experimental methods used were similar to those described earlier (3). In all tests, the culture media contained sufficient biotin (0.01  $\gamma$  per 10 ml.) to satisfy the requirement for that compound. Growth curves were obtained for all compounds which were active as growth factors, and compounds which would not replace methionine or cystine were tested for possible sparing action of the requirements for those amino acids.

*Methionineless Mutant*—The data on the utilization of a number of sulfur-containing compounds by strain 58-161 are given in Table I. It can be seen that the mutant grew in the presence of either L- or D-methionine. The quantity of each isomer necessary for the production of half maximal growth was the same, and the growth curves for the two forms were identical from the origin to the region of half maximal growth. At levels above that necessary for half maximal growth, D-methionine became progressively less effective than L-methionine, and, in this region, the growth curve for D-methionine rose less steeply than that for L-methionine. However, the maximal values of the two curves were the same.

Methionine could be replaced as a growth factor by DL-homocystine, DL-homocysteine, L-cystathionine, and D-allo-cystathionine. The other isomers of cystathionine had only a trace of activity.  $\text{Na}_2\text{S}$  and  $\text{Na}_2\text{S}_2\text{O}_3$  were completely inactive.

Homocystine, as well as autoclaved preparations of homocysteine, showed much less activity than preparations of homocysteine sterilized by filtration under nitrogen. Since formation of homocystine from homocysteine undoubtedly occurs during the 24 hour incubation period used in

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the tests, the value for the quantity of filter-sterilized homocysteine necessary for half maximal growth probably is too high.

*Cystineless Mutant*—As shown in Table II, cystine was the only compound capable of producing growth of strain 58-309. However, the cystine

TABLE I

*Effect of Sulfur-Containing Amino Acids on Growth of Methionineless Mutant*

All cultures were incubated at 30° for 24 hours, and growth was measured by density determinations with the Evelyn photoelectric colorimeter. Total volume of medium for each test, 10 ml. Unless otherwise noted, compounds were added to the minimal medium prior to sterilization by autoclaving.

Compound	Quantity, per 10 ml., for half maximal growth	Remarks
	<i>micromoles</i>	
L-Methionine*	0.071	
D-Methionine*	0.084	At higher concentrations D-methionine is less active than L-methionine
DL-Methionine†	0.071	
DL-Homocystine‡	0.91	No increase in activity on addition of choline
DL-Homocysteine§	1.82	
“ (filter-sterilized)	0.84	
L-Cystathionine§	0.065	
D-Cystathionine§	>9	
L-Allocystathionine§	>9	
D-Allocystathionine§	0.060	
L-Cystine†		No activity or sparing action; largest quantity tested, 42 micromoles
Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> ·5H <sub>2</sub> O		No activity or sparing action; largest quantity tested, 40 micromoles

\* Kindly supplied by Dr. J. S. Fruton.

† Eastman Kodak Company preparation.

‡ Prepared by the method described in (4).

§ Kindly supplied by Dr. V. du Vigneaud. The L-cystathionine contained 0.53 per cent homocystine. The D-cystathionine contained 1 per cent homocystine. The L- and D-allocystathionines contained less than 1 per cent homocystine. For the configuration of the isomers see (5).

requirement was spared to some extent by L-methionine, DL-homocystine, DL-homocysteine, and L-cystathionine.

Maximal growth was not obtained when an excess of L-methionine (20 micromoles per 10 ml.) was added to an amount of cystine (0.1 micromole

TABLE II

*Effect of Sulfur-Containing Amino Acids on Growth of Cystineless Mutant*

All cultures were incubated at 30° for 24 hours. Unless otherwise noted, compounds were added to the minimal medium prior to sterilization by autoclaving. The figures in brackets denote the highest quantity of compound, in micromoles per 10 ml., used in the test.

Compound	Activity in lieu of cystine	Sparing action on cystine requirement, tested in presence of 0.1 micromole of L-cystine
L-Cystine . . . . .	+*	
L-Methionine . . . . .	- [13.4]	+ [20]
D-Methionine . . . . .	- [13.4]	± [10]
DL-Methionine . . . . .	- [20.1]	+ [13.4]
DL-Homocystine . . . . .	- [7.4]	+ [7.4]†
" + choline chloride . . . . .	- [7.4 of each]	
" + DL-serine . . . . .	- [7.4 of each]	
DL-Homocysteine (filter-sterilized) . . . . .	- [7.4]	+ [14.8]
L-Cystathionine . . . . .	- [4.5]	+ [4.5]
D-Cystathionine . . . . .	- [4.5]	- [4.5]
L-Allocystathionine . . . . .	- [4.5]	- [4.5]
D-Allocystathionine . . . . .	- [4.5]	- [4.5]
Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> · 5H <sub>2</sub> O . . . . .	- [4.0]	- [4.0]

\* The quantity of L-cystine required for half maximal growth was 0.13 micromole per 10 ml.

† At levels of 0.02 to 1.1 micromoles per 10 ml., DL-homocystine inhibits the growth of the mutant in the presence of 0.1 micromole of cystine.

TABLE III

*Sparing Action of L-Methionine on Cystine Requirement of Cystineless Mutant*

All cultures were incubated at 30° for 24 hours. Maximal growth of this mutant is produced by approximately 1.5 micromoles of L-cystine per 10 ml. of medium. The extent of growth is given in terms of galvanometer deflections on the Evelyn colorimeter. The galvanometer deflection at maximal growth is 61.

L-Methionine added per 10 ml.	L-Cystine present, in micromole per 10 ml.				
	0	0.0417	0.104	0.208	0.417
micromoles					
0	100	88	84	78	72
0.067	100	84	81.5	77	71.5
0.201	100		78	70.5	66.2
0.67	100	83.5	77.5	70.5	64
2.01	100		75.5	69.5	62.5
6.7	100	80	75.5	69	61
20.1	100	80	73.5		

per 10 ml.) sufficient for approximately half maximal growth. It was necessary to have present about 0.4 micromole of cystine in order to produce maximal growth on the addition of excess methionine (Table III).

The sparing action of L-cystathionine and of filter-sterilized preparations of homocysteine was somewhat less than that of methionine. DL-Homocysteine was found to exert an inhibitory effect on growth when it was used at levels of 0.02 to 1.1 micromoles per 10 ml., but, at levels above 2.2 micromoles per 10 ml., it spared the requirement for cystine. Autoclaved preparations of homocysteine gave results similar to those obtained with homocystine, although inhibition was never observed when filter-sterilized solutions of homocysteine were used.

#### DISCUSSION

From the data obtained with the *methionineless* mutant, it would appear that the biosynthesis of methionine in *Escherichia coli* K-12 occurs by the series of reactions proposed by Horowitz (6) for *Neurospora crassa*.



The data of Lampen *et al.* (7) for several *methionineless* strains of *E. coli* 15 also may be interpreted as evidence for this transformation. If it is assumed that the metabolism of methionine and cystine in mutant and wild type strains of *E. coli* K-12 is identical, except for a single gene-controlled reaction which is lacking in the mutant (8), the mutation by which strain 58-161 was produced caused the loss of an enzyme system necessary for the conversion of L-cystine to L-cystathionine.

It is of interest that the *methionineless* mutant of *Escherichia coli* K-12 utilized D-allo-cystathionine as efficiently as L-cystathionine. Both these compounds can give rise to L-homocysteine by cleavage between the sulfur atom and the 3-carbon chain. Presumably, L-cystathionine is the natural intermediate in the biosynthesis of methionine from cystine in microorganisms, and its production by an appropriate mutant strain of *Neurospora* has been demonstrated (6). However, while the *methionineless* mutant of *Escherichia coli* K-12 could use both L-cystathionine and D-allo-cystathionine in place of methionine, appropriate mutant strains of *Neurospora* could use only L-cystathionine (6).

In the rat, for which methionine is an essential constituent of the diet (9), L-cystathionine is believed to be an intermediate in the biosynthesis of cysteine from homocysteine. L-Cystathionine is cleaved by rat liver preparations to yield L-cysteine (10). Rat liver also is capable of splitting D-allo-cystathionine to D-cysteine (11), but this cleavage would be of little value in the synthesis of cystine *in vivo* since the D isomer is not utilized by the rat for growth (12). It has been found, also, that rat liver prepara-

tions can make D-homocysteine from L-allocystathionine (11) and that L-allocystathionine serves as a dietary source of homocystine (5). Thus, the utilization of L-allocystathionine as a precursor of methionine by the rat appears to be analogous to the utilization of L-cystathionine and D-allocystathionine as precursors of methionine by the *methionineless* mutant of *Escherichia coli*.

The cleavage of any of the isomers of cystathionine to yield cysteine would not be detectable with the *methionineless* mutant. However, such a cleavage seems unlikely in view of the inactivity of these compounds for the *cystineless* mutant.

The *cystineless* mutant, No. 58-309, is the first mutant strain of a micro-organism which has been observed to require cystine and to be unable to satisfy that requirement with methionine, homocysteine, or L-cystathionine (6, 7).<sup>1</sup> The mutant strains of *Escherichia coli* 15, which grow in the presence of cystine or methionine, seem to be able to make cystine from exogenous methionine as well as methionine from exogenous cystine (7). Strain 58-309, a mutant of *E. coli* K-12, probably makes methionine from exogenous cystine although it is not able to carry out the reverse transformation. Therefore, if the metabolism of cystine and methionine in the *E. coli* strains 15 and K-12 is the same, it would appear that the formation of cystine from methionine follows a metabolic pathway different from that used in the conversion of cystine to methionine.

The author wishes to express appreciation to Dr. E. L. Tatum for his valuable advice in the course of these investigations.

#### SUMMARY

A *methionineless* mutant of *Escherichia coli* strain K-12 has been found to grow in the presence of L- and D-methionine, DL-homocystine, DL-homocysteine, L-cystathionine, and D-allocystathionine.

A *cystineless* mutant of *Escherichia coli* strain K-12 has been found to grow only in the presence of L-cystine, L-methionine, DL-homocystine, DL-homocysteine, and L-cystathionine showed a sparing action on the cystine requirement of this mutant.

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## OXIDATION OF HYDROXY-L-PROLINE BY PERIODATE

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Some time ago Carter and Neville reported (1) that hydroxy-L-proline yields formaldehyde on oxidation with periodate. In another laboratory, this oxidation was found to produce a considerably lower yield of formaldehyde than we had obtained.<sup>1</sup> A further study of this reaction was accordingly undertaken. Oxidation of several new samples of hydroxy-L-proline gave lower yields of formaldehyde than those previously obtained. To eliminate interference by possible contaminants, hydroxy-L-proline was purified through the O,N-dibenzoyl derivative. This substance (not previously described) is a very satisfactory derivative for the purification and identification of hydroxy-L-proline. Under the proper conditions, the sodium salt precipitates from the benzoylation mixture in good yields. The free acid is readily purified and reconverted to hydroxy-L-proline. Material purified in this way was oxidized with varying quantities of periodate according to the procedure of Shinn and Nicolet (2). The results clearly indicate that the amount of formaldehyde formed depends critically on the molar ratio of periodate to amino acid. With a ratio of 5 moles of periodate to 1 of amino acid, an insignificant amount of formaldehyde was formed, while proportions of 10:1 and 20:1 of the reactants gave 0.15 and 0.24 moles of formaldehyde, respectively. These variations were reflected by corresponding differences in the rate of oxidation of hydroxy-L-proline as measured by the rate of periodate consumption and the changes in optical activity of the solutions (see Tables I, II, III). These data show that the yield of formaldehyde is much less than that previously reported by us and that hydroxy-L-proline is not likely to interfere with the determination of serine by the methods of Nicolet and Shinn (3) and of Rees (4). In these procedures, the excess of periodate is not sufficient to produce an appreciable amount of formaldehyde from hydroxyproline, as is shown by the observation of Rees (4) that essentially equivalent amounts of volatile aldehyde and ammonia are produced in the oxidation of gelatin hydrolysates by periodate.

No attempt has been made to describe the exact course of the oxidation

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<sup>1</sup> The authors wish to express their appreciation to Dr. A. C. Chibnall for informing them of these results.



of hydroxy-L-proline by periodate. However, the available data show that, once the pyrrolidine ring is ruptured, extensive further oxidation occurs. The optical rotation data indicate that unchanged hydroxy-L-proline is present even after 3 moles of periodate have been consumed. Condensation of formaldehyde with unchanged amino acid or with other degradation products may account for the low yield of dimedon derivative obtained in these studies.

It should be noted that dimedon is readily oxidized by periodate, consuming 3 moles of periodate in 4 hours under the conditions usually employed in the isolation of the formaldehyde dimedon derivative. In calculating the amount of reagent required for condensation with formaldehyde, the amount of excess periodate present in the reaction mixture must be taken into account.

#### EXPERIMENTAL

*Preparation of O,N-Dibenzoylhydroxy-L-proline*—When hydroxyproline is benzoylated in 0.5 N sodium hydroxide, according to the method of Carter and Stevens (5), a low yield of a crystalline solid, the sodium salt of the O,N-dibenzoyl derivative, separates from the reaction mixture. By continuing the benzoylation in a weakly alkaline medium, as recommended by Sørensen and Andersen for the preparation of dibenzoylserine (6), a nearly quantitative yield of the sodium salt is obtained.

In a typical experiment, 4.76 gm. of hydroxyproline were dissolved in 45.0 ml. of cold 20 per cent sodium hydroxide. To this solution were added 20 ml. of benzoyl chloride (4 molar equivalents) in 5 ml. portions. The mixture was kept in an ice bath and shaken vigorously at frequent intervals. During the reaction of the last 10 ml. of benzoyl chloride over a period of 4 to 5 hours, the pH was maintained at 8 to 9 by the addition of 20 per cent sodium hydroxide. After all of the reagent had disappeared, the precipitate was collected, washed twice with small volumes of cold water, dried, and triturated with hot petroleum ether. The white crystalline solid, weighing 12.99 gm. and melting with decomposition at 247–248°, was dissolved in 100 ml. of hot water. On the addition of 5 ml. of concentrated hydrochloric acid, an oil separated and crystallized on standing in an ice bath for several hours. The solid was filtered off, washed thoroughly with cold water, and dried, giving 9.83 gm. (80 per cent yield) of crude product. Recrystallization of this material from ether gave a product melting sharply at 120.5–121.5°.

$C_{19}H_{17}O_5N$  (339.34). Calculated. C 67.26, H 5.05, N 4.13

Found. " 67.46, " 4.85, " 4.16

$[\alpha]_D^{25} = -160^\circ$  (0.49% solution in methanol)

Neutral equivalent, calculated 339, found 333. Saponification equivalent (O-benzoyl group), calculated 339, found 341.

The sodium salt of dibenzoylhydroxy-L-proline dissolves readily in water, although it is only slightly soluble in the benzoylation mixture. The free acid is slightly soluble in cold water or petroleum ether, somewhat soluble in ether and methylcyclohexane, and very soluble in alcohol, acetone, and benzene.

*Regeneration of Hydroxy-L-proline*—6 gm. of dibenzoylhydroxy-L-proline were refluxed with 60 ml. of 10 per cent hydrochloric acid for 10 hours. The mixture was cooled and filtered. Benzoic acid was removed from the filtrate by extraction with ether, and hydrochloric acid by repeated concentration *in vacuo*. The residue was dried *in vacuo* over pellets of potassium hydroxide, dissolved in 10 ml. of water, and passed over a column of Amberlite IR-4. The eluate was concentrated *in vacuo* to a small volume and ethanol added to 90 to 95 per cent concentration, precip-

TABLE I  
*Oxidation of Hydroxyproline with Sodium Metaperiodate*

Time	Periodate consumed per mole hydroxy-L-proline
<i>hrs.</i>	<i>moles</i>
0.0	0.0
0.5	0.53
1.0	0.96
2.0	1.58
4.0	- 2.39
17.5	3.59

itating 1.87 gm. of hydroxy-L-proline (80 per cent yield). An analytically pure sample was obtained by recrystallization from 90 per cent methanol.

$C_8H_9O_3N$  (131.13). Calculated. C 45.80, H 6.92, N 10.69

Found. " 45.94, " 6.74, " 10.63

$[\alpha]_D^{25} = -77^\circ$  (0.324% solution in water)

*Oxidation of Hydroxyproline by Periodate*—The results of Carter and Neville (1) on the rate of oxidation of hydroxyproline by sodium metaperiodate were reproduced. Table I shows the course of the reaction of 30 mg. of hydroxy-L-proline in 100 ml. of 0.01 M sodium metaperiodate (7).

*Effect of Periodate Concentration on Rate of Oxidation*—Hydroxy-L-proline (12 to 13 mg.) was oxidized in 125 ml. flasks with varying amounts of paraperiodic acid in the presence of bicarbonate buffer and arsenite (2, 3) at 30°. In one series, the periodate consumption was determined (7). Water blanks containing arsenite and varying amounts of periodate were also titrated. In another series, the reaction mixture was acidified to methyl red with glacial acetic acid, 30 to 40 ml. of a 0.4 per cent dimedon solution were added, and after 24 hours the formaldehyde derivative,

melting at 189–190°, was filtered off, dried, and weighed. The results are summarized in Table II.

TABLE II

*Effect of Periodate Concentration on Rate of Oxidation and Formation of Formaldehyde from Hydroxy-L-proline*

Weight of hydroxyproline mg.	Molar ratio of periodate to hydroxyproline	Periodate consumed per mole hydroxyproline moles in 1 hr.	Formaldehyde-dimedon derivative		
			Period of oxidation hrs.	Weight mg.	Yield per cent
12.96	5:1		1	0.5	1.7
10.48	5:1		24	0.7	3.0
12.96	10:1		1	4.4	15.2*
16.2	10:1		24	9.0	25.0
12.5	20:1		1	6.6	23.7
12.5	20:1		24	8.0	28.8
13.1	5:1	1.5†			
13.1	10:1	2.5			
13.1	20:1	3.4			

\* This value is low in comparison with the yield reported by Carter and Neville (1). Recalculation of their data gives a yield of 35 to 36 per cent instead of the 60 to 64 per cent previously reported.

† 5 ml. of 0.1 N sodium arsenite were used instead of 10 ml.

TABLE III

*Effect of Periodate on Optical Activity of Hydroxy-L-proline*

Time hrs.	Molar ratio of periodate to hydroxyproline			
	0	5:1	10:1	20:1
0	-0.41			
0.25		-0.07	-0.04	-0.02
0.50		-0.04	-0.02	0.0
0.75		-0.02	0.0	+0.01
1.0	-0.41	-0.01	0.0	+0.02
3.5		*	*	+0.05
24.0	-0.41	+0.05	+0.05	+0.05

\* The solution had turned dark yellow; on standing the color gradually became less intense.

*Change in Optical Activity of Hydroxy-L-proline Solutions during Periodate Oxidation*—The rate of change in optical activity with varying concentrations of periodate was determined. Into each of three 10 ml.

volumetric flasks was pipetted 1 ml. of a solution of hydroxy-L-proline containing 52.4 mg. or 0.4 mm per ml. Aliquots of a solution of sodium metaperiodate equivalent to 2, 4, and 8 mm of periodate were added. The solutions were diluted to volume with water and transferred to 1 dm. polarimeter tubes. A record of the rotations observed over a period of 24 hours is given in Table III.

In another experiment, 2.567 gm. (3 molar equivalents) of sodium metaperiodate were added to 0.524 gm. of hydroxy-L-proline dissolved in 85 ml. of 0.1 N sodium arsenite solution. Within 1 hour, all of the reagent had reacted and the observed rotation had changed from  $-0.47^{\circ}$  to  $-0.15^{\circ}$ . This reading remained constant until an additional 3 molar equivalents of periodate were added, when the rotation changed from  $-0.15^{\circ}$  to  $+0.07^{\circ}$  within a half hour.

#### SUMMARY

Hydroxy-L-proline has been purified and characterized through the O,N-dibenzoyl derivative. An investigation of the reaction of pure hydroxy-L-proline with periodate has shown that extensive oxidation occurs. Among the degradation products are a dextrorotatory substance and formaldehyde. One of the rate-determining factors is the ratio of periodate to amino acid; the greater the ratio the more rapid the uptake of periodate, the greater the change in optical activity, and the higher the yield of formaldehyde. Hydroxy-L-proline is not likely to interfere with the determination of serine by periodate oxidation.

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# A SIMPLIFIED PROCEDURE FOR THE PREPARATION OF CYTIDYLIC ACID AND DIAMMONIUM URIDYLATE FROM YEAST NUCLEIC ACID\*

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Previously published methods for the preparation of the pyrimidine nucleotides are relatively involved procedures requiring either repeated crystallization of the brucine salts (1) or fractionation of the free acids by their differential solubility in pyridine (2). Although the yields obtained by the former method were not published, we have isolated less than 2 per cent of the original nucleic acid as crystalline diammonium uridylate by this procedure. Yields of 5.6 and 4 per cent of the original nucleic acid as crystalline cytidylic and "nearly" crystalline uridylic acids respectively were reported for the pyridine extraction procedure. Other workers, however, have found lower yields by the latter method (3) and in our own laboratory the yield of crystalline cytidylic acid isolated from a sample of yeast nucleic acid by the pyridine method was 1.2 per cent. Although the results of bioassays for cytidylic and uridylic acids indicate that the concentrations of these constituents in ribonucleic acid are less than those expected from a tetranucleotide structure (4), it is evident from the small yields mentioned that the isolation procedures are highly inefficient.

The extent to which nucleosides or nucleotides are produced by alkaline hydrolysis of yeast nucleic acid depends on the concentration and type of alkali used and on the temperature at which the hydrolysis is carried out (1, 5). The published procedures for the preparation of nucleotides have avoided hydrolysis to nucleosides by the use of ammonia at 115–120° in an autoclave (5) or alternately by the use of stronger alkali at room temperatures for relatively long periods of time (6, 7). In view of the difficulties inherent in the former procedure and the uncertainty regarding the extent of hydrolysis in the latter, it seemed desirable to find conditions in which nucleic acid could be converted to nucleotides by refluxing in the presence of a relatively strong base. Because of the insoluble nature of some of the barium salts of the nucleotides (8, 9), the use of barium hydroxide as a hydrolyzing agent was studied. In order to find optimum conditions

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for the preparation of the nucleotides, the concentration of alkali was determined which could be refluxed with nucleic acid for 2 hours without the liberation of appreciable amounts of inorganic phosphate. The resulting hydrolysates as well as those prepared by treatment with dilute mineral acid (8) were fractionated with 12-phosphotungstic acid, which has been shown to effect a nearly quantitative separation of cytidylic acid or cytidine from uridylic acid or uridine (4). The present paper records the results in which these procedures have been applied to the preparation of cytidylic acid and diammonium uridylate from yeast nucleic acid.

#### EXPERIMENTAL

*Hydrolysis of Yeast Nucleic Acid by Barium Hydroxide*—A series of preliminary experiments employing different amounts of barium hydroxide were performed to determine the optimum conditions for hydrolysis to nucleotides. Two criteria of the extent of hydrolysis were employed; namely, (a) the amount of inorganic phosphate liberated and (b) the growth activity of the resulting hydrolysates for the pyrimidine-deficient *Neurospora* mutant No. 1298. 2 gm. samples of commercial yeast nucleic acid were refluxed for 2 hours in the presence of 20 ml. of water and sufficient solid barium hydroxide to give solutions that were 0.5 N, 0.55 N, 0.6 N, 0.7 N, and 0.8 N with respect to barium ion. The samples were acidified with enough hydrochloric acid to dissolve the insoluble barium salts, barium ion was removed, as the sulfate, and aliquots of the resulting solutions were analyzed for inorganic phosphate (10) and assayed for growth activity (4).

The phosphate analyses showed that from 2 to 3 per cent of the total nucleic acid phosphorus was liberated as inorganic phosphate over the normality range from 0.5 to 0.7 N and that 6.5 per cent was formed in the 0.8 N solution. Whether the inorganic phosphate was derived entirely from a single nucleotide or in part from each of the purine and pyrimidine nucleotides known to be present could not be determined from the data obtained. If the assumption was made, however, that one-half of the inorganic phosphate was formed from pyrimidine nucleotides and that a corresponding amount of pyrimidine nucleoside resulted, the growth activity due to cytidylic acid or uridylic acid varied from 22 to 26 per cent over the normality range from 0.5 N to 0.7 N and increased to about 32 per cent for the 0.8 N solution. If a larger proportion of the inorganic phosphate were derived from pyrimidine nucleotides in the more alkaline solutions, the growth activity found would be due to a larger proportion of pyrimidine nucleosides and the amount of pyrimidine nucleotides present would be correspondingly reduced. In a few additional experiments in which stronger alkali or a longer period of hydrolysis was employed, additional amounts of inorganic phosphate were formed without further increasing growth activity for the mold.

On the basis of the above-mentioned experiments, it seemed desirable for the isolation of the nucleotides to select conditions of hydrolysis intermediate between the extremes mentioned. Accordingly a 10 per cent nucleic acid suspension in 0.6 N barium hydroxide was employed. Under these conditions a large fraction of the original nucleic acid is converted to relatively insoluble barium salts which could be removed by filtration and used for the preparation of diammonium uridylate and cytidylic acid. Alternately the suspension could be treated directly with enough sulfuric acid to convert the barium salts to barium sulfate and a solution of the nucleotides. The latter were then fractionated by the same procedure used for the barium-insoluble salts. The latter procedure, while not advantageous for the preparation of cytidylic acid, gave the best yield of diammonium uridylate.

*Isolation of Pyrimidine Nucleotides from Insoluble Barium Salts*—50 gm. of yeast ribonucleic acid (B. L. Lemke Company) were added to a beaker containing 500 ml. of boiling 0.6 N barium hydroxide solution. The mixture was carefully heated to boiling to avoid frothing, was boiled for 2 hours, and then cooled in ice. The granular precipitate of barium salts of the nucleotides was filtered off by suction and was washed with a little cold barium hydroxide solution. The solid was suspended in about 200 ml. of hot water and the solution was acidified with enough 6 N sulfuric acid to provide a slight excess of sulfate ions (blue with Congo red paper). The resulting solution of nucleotides was filtered from insoluble barium salts through a pad of Hyflo Super-Cel and the barium sulfate was washed twice by suspension in boiling water. The washings were combined with the original filtrate, which was cooled in ice and refiltered from a small amount of insoluble material which formed. To the warm solution 20 gm. of 12-phosphotungstic acid (11) dissolved in the minimum volume of water necessary for solution were added slowly until no further precipitation took place. After cooling in ice the heavy precipitate was filtered onto a pad of Hyflo Super-Cel, and the filtrate was concentrated *in vacuo* to a volume of about 200 ml. 7.5 ml. of concentrated sulfuric acid were added to make the solution approximately 1 N, and then 45 gm. of 12-phosphotungstic acid were added to the hot solution. After immediate filtration through Hyflo Super-Cel to remove the last traces of the phosphotungstates of the purine nucleotides, the solution was allowed to stand at room temperature for several hours and cooled in the refrigerator overnight. Cytidylic acid phosphotungstate separated as a gelatinous precipitate which was best removed by centrifugation in the cold. The solid was washed once in the centrifuge with a little ice-cold 1 N sulfuric acid and was used for the preparation of cytidylic acid. As cytidylic acid phosphotungstate is quite soluble in 1 N acid at room temperature, it is necessary to keep the solution as cold as possible during centrifugation.



yield of crystalline cytidylic acid by this procedure was less than that given above, being from 2 to 2.8 per cent of the original nucleic acid.

*Diammonium Uridylate*—The supernatant liquid and washings from the cytidylic acid phosphotungstate were freed of phosphotungstic acid and converted to diammonium uridylate as previously described. The yield in two experiments varied from 5.6 to 6.2 per cent of the original nucleic acid.

*Hydrolysis with Mineral Acids*—The fractionation procedure described above was applied to hydrolysates of nucleic acid obtained after hydrolysis for 2 hours with 0.4 N sulfuric acid, nitric acid, and hydrochloric acid (8). The free purines which are produced under these conditions were removed with silver and the resulting filtrates fractionated with phosphotungstic acid after removal of silver ion. Cytidylic acid was obtained in from 3.3 to 3.6 per cent yield, and diammonium uridylate in about 3.8 per cent yield. As compared to the yields previously mentioned, this procedure was not advantageous.

#### SUMMARY

Procedures are described for the preparation of cytidylic acid in a yield of 4.4 per cent and of diammonium uridylate in yields of from 4.6 to 6.2 per cent of the original yeast nucleic acid. The procedure is based on the hydrolysis of the nucleic acid with 0.6 N barium hydroxide and the fractionation of the phosphotungstates of the purine nucleotides and of cytidylic acid in weakly and strongly acid solutions under conditions in which uridylic acid is not precipitated. The same fractionation procedure was applied to hydrolysates obtained after hydrolysis with mineral acid, but the yields of the pyrimidine nucleotides were not as large as those obtained after alkaline hydrolysis.

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## PARTIAL PURIFICATION OF THE VASOCONSTRICTOR IN BEEF SERUM\*

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The demonstration that plasma from persons with hypertension is more vasoconstrictor than normal plasma would be direct evidence supporting a humoral mechanism for the disease. This necessitates evaluating effects of vasoactive substances which may be newly formed in the blood subsequent to withdrawal of the sample. The chief, and perhaps only, substance of importance in this regard is the vasoconstrictor appearing during the clotting or defibrination of blood.

Mainly with the purpose of removing this obstacle to the study of differences between plasmas from hypertensive and normal subjects, attention has been directed to the isolation and identification of the substance in serum responsible for its vasoconstrictor activity as measured in the vessels of the isolated rabbit ear. There are other reasons for establishing the identity of this substance, among which may be mentioned its probable rôles in hemostasis and intravascular clotting.

Since the problem of the serum vasoconstrictor is well over 50 years old, an extensive literature dealing with the subject is available (1-5) which will not be reviewed again here. Most of the papers discuss the pharmacological properties of the unpurified substance, its relative stability, and solubility. The results published show a high degree of inconsistency, reflecting the inability of previous workers to obtain quantitative information from their assay methods.

This report summarizes the results of work on the purification and properties of the serum vasoconstrictor in quantitative terms. The method of assay, consisting of perfusion of the blood vessels of isolated rabbit ears (6, 7), has proved capable of giving reproducible results within 10 to 20 per cent in the same preparation, with checks over several months within 25 to 50 per cent. A five-step purification procedure has been developed which yields a preparation of the vasoconstrictor material about 200 times as active, on a dry weight basis, as that in serum itself, with recovery of 25 to 50 per cent of the original activity in serum. The dry preparation can be stored without loss of activity, apparently indefinitely. At every stage of

\* A preliminary report of this work has been published (*Federation Proc.*, 6, 184 (1947)). The expense of this work was defrayed in part by a grant from the United States Public Health Service, Committee on Cardiovascular Disease.

the purification before the fifth, considerable instability of the substance is observed.

The method of purification described in detail below represents a summary of experience obtained with more than 60 runs, involving 900 liters of serum collected from almost 2 tons of beef blood.

The advantages of the procedure are that it is rapid, inexpensive, and gives preparations with reasonably constant properties. It permits purification without serious losses and provides a stable preparation suitable for accumulation for further purification. The procedure is adaptable to large quantities of serum without elaborate equipment.

#### EXPERIMENTAL

*Collection of Serum*—Clotted whole beef blood, obtained from the slaughter-house in a lot of 45 liters (eight buckets), is placed in a cold room as soon as received. The eight clots yield 15 to 20 liters of serum<sup>1</sup> of which 15 liters are processed at a time. The fluid is centrifuged in the cold. The collection of 15 liters of serum takes 2 days.

*Step 1. Precipitation of Proteins*—The proteins are precipitated from the serum, after adjusting the pH to 4.5 to 5.0, by means of 95 per cent ethyl alcohol. The pH adjustment has been found necessary for two reasons. First and most important is that large losses of activity result from concentration of solutions above pH 7. Second, more effective removal of proteins is obtained at the lower pH. The 15 liters of serum are processed in three 5 liter batches at this step.

5 liters of serum are adjusted to pH 4.5 to 5.0 with 130 ml. of 2 N HCl. After the addition of 6.7 liters of 95 per cent ethyl alcohol, the mixture is stirred well and left in the cold room overnight. The protein precipitate is removed by suction filtration, giving 9 to 9.5 liters of filtrate.

The combined filtrates (27 to 28 liters) are then concentrated at reduced pressure under nitrogen to about 300 ml., the temperature being kept below 40°. With the equipment at our disposal, this operation required 20 to 25 hours. Losses of activity are found to be increased with increase in distillation time. To control foaming, 10 ml. portions of 2-ethylhexanol are added as required.

*Step 2. Removal of Acetone-Insoluble Impurities*—To the 300 ml. of concentrate, 10 volumes of acetone are added. The mixture is shaken, then

<sup>1</sup> The serum is most conveniently collected by means of an apparatus which permits the eight clots to drain simultaneously. This apparatus consists of a 24 gallon can, the bottom of which has been removed. The can rests on a board with a circular opening covered by  $\frac{1}{4}$  inch rectangular mesh galvanized screen, in turn covered by gauze. The expressed fluid falls into the can cover which rests on top of a 9 liter glass jar. The cover has two small holes drilled through its center.

stirred mechanically at room temperature until the insoluble phase becomes solid. This takes 30 to 60 minutes. The mixture is then chilled in the cold room overnight.

The clear, light yellow supernatant solution, which contains about 90 per cent of the active principle, is decanted from the solid (consisting mainly of inorganic salts, phosphatides, carbohydrates, and amino acids) and evaporated under reduced pressure in a bath kept below 40° to a volume of 80 to 100 ml. The troublesome foaming which occurs as the last of the acetone evaporates is controlled by increasing the pressure temporarily.

*Step 3. Extraction of Inactive Substances with Chloroform*—The mixture, which consists at this point of a yellow emulsion with floating dark brown oil, is transferred with the aid of an equal volume of chloroform and a few ml. of ethanol to a 500 ml. separatory funnel.

From here on, all operations are carried out in a cold room at 5°.

Shaking the two phases results in a stiff emulsion, which occasionally breaks overnight. More often it is necessary to add 10 gm. of solid ammonium sulfate and several ml. of methanol. After standing overnight, this results in a sharp separation of the phases into an upper, clear, light yellow aqueous layer, and a lower, brown chloroform layer. It has rarely been found necessary to resort to centrifugation.

The active principle is entirely in the aqueous phase,<sup>2,3</sup> although the presence of some active material in the chloroform may be masked by the fact that the other substances in this layer are intensely vasodilator when tested in the rabbit ear.

*Step 4. Extraction of Active Principle with Butanol*—The aqueous layer is adjusted to pH 5.9  $\pm$  0.3 with 5 N NaOH, 5 to 6 ml. being required. This adjustment is not critical. The solution is then saturated with ammonium sulfate by the addition of 0.6 gm. of the salt per ml., and subsequently extracted three times by shaking several minutes with equal volumes of butanol. The combined butanol extracts are left overnight at -10° and then decanted.

*Step 5. Precipitation of Active Principle with Dilituric Acid*—To the butanol extract, 150 ml. of a solution of dilituric acid (5-nitrobarbituric

<sup>2</sup> On two or three occasions, a large fraction of the vasoconstrictor substance has gone into the chloroform phase. This has been attended by a concomitant large increase in the volume of the chloroform layer with corresponding decrease in the volume of the aqueous layer. The effect is attributed to a change in the solvent properties of the chloroform resulting from the large quantity of dissolved material. Increasing the aqueous volume of the initial extraction or employing an additional aqueous extraction of the chloroform corrected this aberration.

<sup>3</sup> The aqueous phase, which is at pH 2.9 to 3.2, has a concentration of activity 50 to 100 times greater than that of serum. The active material is not stable in this solution, over 55 per cent having been observed to disappear in 10 days at 5°.

acid) in methanol (prepared by dissolving 12 gm. of the acid in 800 ml. of absolute methanol at the boiling point, cooling, and filtering) are added, resulting in an immediate precipitation of colorless solid.

After the mixture has stood overnight at 5°, a sample of the supernatant solution is tested for completeness of precipitation by the addition of a small quantity of the methanolic solution of the acid. If more precipitate forms, another 10 ml. of the dilituric acid solution are added to the mixture. The procedure is repeated until complete precipitation is obtained, which is usually indicated by a faint yellow color.

The precipitate is collected by centrifugation and is then washed three times with small quantities of cold methanol to remove excess dilituric acid and butanol. The almost colorless solid is then dried in a desiccator by prolonged evacuation. The precipitate contains over 95 per cent of the activity present in the butyl alcohol extract. The yield from 15 liters of serum is about 2 gm. of this dry precipitate, containing 25 to 50 per cent of the activity found in the original serum.

*Method of Assay*—The assay is made by injection into the vessels of the isolated rabbit ear prepared as described by Page (6, 7). Solutions of the unknown are diluted until 0.2 ml. gives a response identical with that of 0.2 ml. of a standard control solution of the vasoconstrictor substance. The standard is a solution of material from Step 5 pooled from ten runs. A concentration of 1.2  $\gamma$  per ml. has been arbitrarily designated as containing 1 unit of activity. On this basis, the rabbit ear preparation shows the responses to other vasoactive substances recorded in Table I. The reproducibility of the ear preparation responses has been found to be 10 to 20 per cent. The greater range shown for some of the substances in Table I is a result of the inability to obtain constrictions with them identical with those of serum vasoconstrictor. The composition of the perfusion fluid in gm. per liter is as follows: NaCl 8.2, KCl 0.84,  $MgCl_2 \cdot 6H_2O$  0.06,  $CaCl_2 \cdot 2H_2O$  0.04, glucose 1.0,  $NaHCO_3$  0.40. In addition, 8 ml. of 1 M  $K_2HPO_4$  and 2 ml. of 1 M  $KH_2PO_4$  are added per liter.

*Properties of Purified Preparation*—The range of activity of the purified preparation is 500 to 1000 units per mg. The bulk of the solid is composed of ammonium diliturate; analysis by direct nesslerization shows 60 to 85  $\gamma$  of  $NH_3$  per mg. The results of other color tests on this material are shown in Table II.

The heat stability of the substance in solution appears to be considerably increased by the fifth stage in purification. For example, neither heating at 100° for 5 hours at a concentration of 1 mg. per ml. nor standing for 1 month at room temperature at this concentration resulted in significant inactivation. Whether this increased stability is due to the stabilizing influence of the dilituric acid or to the removal of reactive impurities is not known.

The material does not show appreciable solubility in organic solvents. However, it can be dissolved in boiling water to the extent of 8 mg. per ml., but readily crystallizes on cooling. The solubility at room temperature is

TABLE I

*Activity of Several Vasoconstrictor Substances in Perfused Ear Vessel Preparation, Based on Arbitrarily Defined Unit of Activity*

Substance	Units per mg. at 1 mg. per ml.
Epinephrine hydrochloride.....	50,000-100,000
Tyramine.....	500- 1,000
Histamine phosphate.....	3,000- 4,000
Tryptamine hydrochloride.....	5,000

TABLE II

*Color Reactions of Purified Material of Step 5*

Color reaction	Standard*	Comparable color obtained per mg. ppt. from Step 5
		$\gamma$ of standard
Gerngross-Voss-Herfeld for <i>p</i> -hydroxyphenyl groups.....	Tyrosine	2-3
Pauly diazo for imidazoles.....	Histidine	2-3
Sakaguchi for guanidino groups.....	Arginine	<2
Folin-Ciocalteu for phenols, indoles, etc.....	Tyrosine	16-21
Ferrie chloride.....	Epinephrine	<2

\* The quantity of standard substance used for color development is 10  $\gamma$  for all reactions except the Gerngross-Voss-Herfeld reaction which requires 50  $\gamma$ .

TABLE III

*Dialysis of Serum Vasoconstrictor Against 2 Volumes of 0.01 M Phosphate Buffer, pH 7.4, at 5°, without Stirring*

Time  hrs.	Concentration	
	Inside casing	Outside casing
	units per ml.	units per ml.
0	600	0
24	300	150
48	200	200
48; buffer solution replaced after 24 hrs.	125	75

about 2 mg. per ml.; at 5°, slightly more than 1 mg. per ml. Repeated recrystallization from hot water can be effected without any appreciable change in the activity of the precipitate. High concentrations of activity

in aqueous solution are easily obtained by removing the dilituric acid as its insoluble magnesium salt (8) with  $MgCl_2$  or  $MgSO_4$ . No loss of activity is encountered in this procedure, but the use of basic magnesium salts results in considerable inactivation (30 to 50 per cent).

Studies on the chemical inactivation will be reported in a subsequent publication.

*Some Properties of Active Principle As Found in Serum*—Variable effects have been observed with regard to stability of the substance in serum, but, in general, centrifuged serum loses about half its activity in 5 days at 5°.

Considerable disagreement exists concerning the dialyzability of the serum vasoconstrictor. Quantitative data measuring this property are presented in Table III. The results indicate that the substance not only di-

TABLE IV  
*Concentration and Recovery of Beef Serum Vasoconstrictor*

Fraction	Original serum	Dry weight
	units per l.	units per mg.
Serum.....	300,000	4
Ethanol filtrate, Step 1.....	240,000	10
Aqueous concentrate, 300 ml, Step 1.....	150,000	10
Acetone supernatant, Step 2.....	130,000	90
Chloroform-washed aqueous phase, Step 3....	120,000	200
Butanol extract, Step 4.....	100,000	
Dilituric acid ppt., Step 5.....	100,000	750

alyzes very rapidly and completely, but that it is not appreciably adsorbed by serum proteins.

The range in activity found in beef serum is 100 to 600 units per ml.

#### DISCUSSION

A summary of the purification process with regard to concentration and recovery of activity is presented in Table IV.

The question of the presence of multiple substances being responsible for the vasoconstrictor effect of serum (2, 9) is not answered by this study. However, the uniformity of behavior of the active material throughout the purification procedure, as well as the high recovery of activity from serum, argues for the principal measured effect as being due to a single substance. Certain physical properties, such as dialysis, are also consistent with this view. As can be seen from Table III, the rate of dialysis is approximately the same, starting with fresh serum and with serum from which half the activity has already been removed by dialysis.

If the assumption is made that the effect is due to a single substance,

then the data presented in Tables I and II rule out epinephrine, tyramine, histamine, and tryptamine on the evidence of activity-color ratios. For example, the value for the activity of epinephrine is 50,000 to 100,000 units per mg. The comparative color given by epinephrine in the ferric chloride test is 1000  $\gamma$  of standard per mg., since epinephrine is used as the color standard. The ratio of activity in units per mg. to color in micrograms of standard per mg. for this test is therefore 50 to 100 for epinephrine. For the purified material of Step 5, the ratio is  $>350$ . The fact that this activity-color ratio is much *greater* with the purified material than with epinephrine, coupled with the assumption that activity is due to a single substance, makes it impossible for epinephrine to be that substance.

A similar argument may be applied to the other compounds with the Folin-Ciocalteu reaction for tyramine and tryptamine and the Pauly reaction for histamine.

Further experiments aimed toward ultimate isolation and characterization of the active material are in progress.

The authors wish to thank Mr. John M. Means, Miss Martha Bender, and Miss Elizabeth Hunt for valuable assistance.

#### SUMMARY

A five-step purification is described which results in a 200-fold concentration, on a dry weight basis, of the serum vasoconstrictor, a substance which appears during the clotting or defibrination of blood. The recovery of activity is 25 to 50 per cent of that in the original serum. The principal advantage of this procedure is that it yields a preparation with enhanced stability, permitting accumulation for further purification.

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# BIOCHEMICAL TRANSFORMATIONS AS DETERMINED BY COMPETITIVE ANALOGUE-METABOLITE GROWTH INHIBITIONS

## VIII. AN INTERRELATIONSHIP OF METHIONINE AND LEUCINE\*

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After Harris and Kohn (1) first reported the prevention of the toxicity of norleucine by methionine for *Escherichia coli*, Porter and Meyers (2) found that norleucine in preventing growth of *Proteus morganii* was competitive with methionine. Lampen and Jones (3) recently demonstrated that norleucine prevented competitively the functioning of methionine in *Escherichia coli*.

The technique of *inhibition analysis*, which has been used successfully in determining the biosynthetic sequence of metabolites in several systems (4-6), has been employed in the present investigation of the toxicity of norleucine for *Escherichia coli*. Of the known naturally occurring amino acids, methionine alone prevents competitively the toxicity of norleucine; however, leucine but not the corresponding keto acid prevents the toxicity of norleucine in such a manner as to suggest that methionine functions in the biosynthesis of leucine, probably in the amination of  $\alpha$ -keto- $\gamma$ -methylvaleric acid. The ability of isoleucine and valine to replace leucine in preventing the toxicity of the inhibitor suggests that the biosyntheses of these amino acids also involve the enzymatic reaction which is blocked by norleucine. Details of these and related findings are presented below.

### EXPERIMENTAL

*Testing Methods*—For tests with *Escherichia coli* the medium was prepared as follows:  $\text{Na}_2\text{SO}_4$ , anhydrous, 1 gm.,  $\text{NH}_4\text{Cl}$  1 gm.,  $\text{K}_2\text{HPO}_4$  0.8 gm.,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  80 mg., glucose 2 gm., and  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  20 mg. were dissolved in 100 cc. of distilled water, steamed for 5 minutes, and filtered after cooling to room temperature. For assays, 5 cc. of this double strength basal medium were added to the test materials contained in 5 cc. of water. The tubes were capped, autoclaved 10 minutes at 15 pounds steam pressure, cooled, and inoculated. Times and temperatures of incubation are given in each table.

\* For the most part from a thesis submitted by W. M. Harding to The University of Texas in partial fulfillment of the requirements for the degree of Master of Arts, August, 1946.

The organism was a previously described (4) strain of *Escherichia coli*, maintained by monthly transfers on 1 per cent glucose, 1 per cent yeast extract, and 1.5 per cent agar but grown for at least two daily transfers on the inorganic salts-glucose medium described above before use in the tests reported in Tables I, II, IV, and V. The data in the other tables were obtained by use of this same organism grown for at least eight daily transfers in the inorganic salts-glucose medium supplemented with 1 to 10 mg. of DL-methionine per 10 cc. Such "training" of the organism did not result in tests which were qualitatively different from tests obtained with the "untrained" strain. The inoculum for a test was prepared by centrifuging a 10 cc. daily transfer after 14 to 18 hours growth, washing the cells in 10 cc. of 0.9 per cent sodium chloride solution, and resuspending in 10 cc. of the saline solution. A drop of a 1:100 dilution of this suspension was used to inoculate each tube of the test.

The basal medium used for the tests with *Lactobacillus pentosus* was that of McMahan and Snell (7) with the omission of leucine, isoleucine, valine, methionine, norleucine, norvaline, hydroxyproline, adenine, guanine, and xanthine and the doubling of the amount of Salts A. This medium was not sufficient for growth, but abundant growth was obtained in 24 hours when leucine, isoleucine, and valine were added. If these three amino acids were omitted and  $\alpha$ -keto- $\gamma$ -methylvaleric acid,  $\alpha$ -keto- $\beta$ -methylvaleric acid, and  $\alpha$ -hydroxyisovaleric acid (1 mg. each per 10 cc.) were included in the medium, good growth occurred in 2 days. Before the organism was used in a test it was grown in five successive 48 hour transfers in the latter medium, after which time good growth was obtained in 30 to 36 hours in this medium.

The tests prepared for inoculation with *Lactobacillus pentosus* were autoclaved 15 minutes before inoculation. The organisms to be used for inoculation were centrifuged and washed as described for *Escherichia coli*. The washed suspension was diluted 1:20 and 1 drop of this suspension was used to inoculate each tube.

**Results**—Of the amino acids normally occurring in proteins, only methionine, leucine, isoleucine, valine, glutamic acid, and threonine prevented the toxicity of norleucine for *Escherichia coli* to any significant extent, their respective degrees of effectiveness decreasing in the order named.

As is shown in Table I, methionine reverses competitively the inhibitory effect of norleucine on *Escherichia coli*. The molar ratio of DL-norleucine to DL-methionine just necessary to prevent the growth of the organism (the antibacterial index) (8) is within the range of 30 to 100 under the testing conditions employed. The antibacterial index determined with the addition of 100  $\gamma$  of L-leucine per 10 cc. of medium was approximately 10 times the ratio obtained in the absence of exogenous leucine.

Separate experiments indicated that leucine, isoleucine, valine, or any four mixtures which can be made with leucine, isoleucine, and valine did not competitively prevent the toxicity of norleucine.

However, as is indicated in Table II, increasing concentrations of leucine

TABLE I

*Effect of Leucine on Norleucine Toxicity*

Test organism, *Escherichia coli*; incubated 15 hours at 38-39°.

DL-Norleucine	DL-Methionine	Galvanometer readings*	
		Without L-leucine	With L-leucine (100 $\gamma$ per 10 cc.)
$\gamma$ per 10 cc.	$\gamma$ per 10 cc.		
0	0	52	55
10	0	10	53
30	0	2	16
100	0		2
0	3	54	59
30	3	52	59
100	3	32	58
300	3	5	50
1,000	3	2	8
0	10	54	54
100	10	56	58
300	10	22	57
1,000	10	2	45
3,000	10		18
0	30	56	55
300	30	60	59
1,000	30	10	54
3,000	30	2	44
10,000	30		38
30,000	30		8
0	100	63	60
1,000	100	55	60
3,000	100	14	53
10,000	100	2	48
30,000	100		14
Antibacterial index.....		30-100	300-1000

\* A measure of culture turbidity; distilled water reads 0, an opaque object 100.

above 10  $\gamma$  per 10 cc. had only slight effects until relatively high concentrations of leucine above 300  $\gamma$  per 10 cc. were employed. Above that concentration, the effect of leucine appeared to be competitive at a ratio of norleucine to leucine of approximately 0.3. This effect at high concentra-

tions suggested the possibility of contamination of the L-leucine with methionine. An assay for methionine in the leucine by the method of Stokes *et al.* (9) with *Streptococcus faecalis* R indicated that the quantity of methionine present was insufficient to account totally for the effects shown in Table II. However, DL-leucine (synthetic and presumably free of methionine) was tested against norleucine in the same manner as L-leu-

TABLE II

*Effect of Increasing Concentrations of Leucine on Norleucine Toxicity*

Test organism, *Escherichia coli*; incubated 14 hours at 37-38°.

DL-Norleucine	L-Leucine	Galvanometer reading
$\gamma$ per 10 cc.	$\gamma$ per 10 cc.	
0	0	53
10	0	16
30	0	1
0	10	57
10	10	41
30	10	10
100	10	1
0	30	56
10	30	44
30	30	28
100	30	1
0	100	57
10	100	54
30	100	32
100	100	2
0	300	52
30	300	50
100	300	8
300	300	2
0	1000	54
30	1000	51
100	1000	38
300	1000	2
0	3000	54
300	3000	51
1000	3000	6

cine, and at high concentrations DL-leucine prevented the toxicity of norleucine in a manner similar to that of L-leucine; however, the effect of DL-leucine at higher concentrations cannot be described as competitive in that 10-fold increases in concentration of DL-leucine (300 to 3000  $\gamma$  and 1000 to 10,000  $\gamma$  per 10 cc.) required only 3-fold increases in concentration of norleucine to inhibit growth of the organism in several tests.

The effect of increasing concentrations of leucine on the toxicity of nor-leucine is further shown in Table III which indicates the effects of 100 and 3000  $\gamma$  per 10 cc. of DL-leucine on the antibacterial index. In this and other tests, low concentrations of leucine (30 to 300  $\gamma$  per 10 cc.) increase the antibacterial index from 3- to 10-fold. Higher concentrations of leucine further prevented the toxicity of norleucine, and the effect was not completely "diluted out" by high concentrations of methionine.

In Tables IV and V, the effects of DL-valine and DL-isoleucine on nor-

TABLE III

*Effect of Increasing Concentrations of Leucine on Inhibition Index*

Test organism, *Escherichia coli*; incubated 16 hours at 37-38°.

DL-Norleucine $\gamma$ per 10 cc.	DL-Methionine $\gamma$ per 10 cc.	Galvanometer readings		
		Without DL-leucine	With DL-leucine (100 $\gamma$ per 10 cc.)	With DL-leucine (3000 $\gamma$ per 10 cc.)
0	10		48	46
300	10		44	46
1,000	10		13	46
3,000	10		5	38
10,000	10			5
0	30	52	50	44
300	30	45	47	46
1,000	30	12	38	46
3,000	30	5	7	46
10,000	30		5	21
30,000	30			5
0	100	54	50	45
1,000	100	46	50	45
3,000	100	5	35	44
10,000	100		5	38
30,000	100			8
Antibacterial index..		30 Ca.	100 Ca.	300-1000 Ca.

leucine toxicity are shown. The addition of either amino acid to the medium resulted in a higher antibacterial index by approximately 3-fold.

The relative effects of leucine, isoleucine, and valine are indicated in Table VI. At a concentration of 200  $\gamma$  per 10 cc. and in the presence of 10  $\gamma$  per 10 cc. of DL-methionine, which was used for the purpose of preventing any possible precursor effects, DL-leucine was slightly more effective than DL-isoleucine, which in turn was slightly more effective than DL-valine. This order of activity in other tests was often more pronounced. Increased concentrations of DL-isoleucine or DL-valine did not give an effect

similar to that given by increased concentrations of DL-leucine. However, a mixture of the two was able to replace leucine in producing this effect.

In separate experiments,  $\alpha$ -keto- $\gamma$ -methylvaleric acid,  $\alpha$ -keto- $\beta$ -methylvaleric acid, or  $\alpha$ -hydroxyisovaleric acid had no effect on the toxicity of norleucine for *Escherichia coli* in the inorganic salts-glucose medium.

In the modified amino acid medium containing  $\alpha$ -keto- $\gamma$ -methylvaleric acid,  $\alpha$ -keto- $\beta$ -methylvaleric acid, and  $\alpha$ -hydroxyisovaleric acid, nor-

TABLE IV

*Effect of Valine on Norleucine Toxicity*

Test organism, *Escherichia coli*; incubated 14 hours at 37-38°.

DL-Norleucine	DL-Methionine	Galvanometer readings	
		Without valine	With DL-valine (200 $\gamma$ per 10 cc.)
$\gamma$ per 10 cc.	$\gamma$ per 10 cc.		
0	0	52	55
10	0	10	53
30	0	1	16
100	0		1
0	3	54	51
30	3	52	59
100	3	32	49
300	3	5	45
1,000	3	2	5
0	10	54	57
100	10	56	56
300	10	22	53
1,000	10	2	34
3,000	10		4
0	100	63	62
1,000	100	55	54
3,000	100	14	46
10,000	100	0	2
Antibacterial index.....		30-100	100-300

leucine at a concentration of 1 mg. per 10 cc. almost completely prevented growth of *Lactobacillus pentosus*. As is indicated in Table VII, the inhibition was prevented in a competitive manner by methionine, and at a ratio of norleucine to methionine of approximately 3000, complete inhibition of growth was just attained. Leucine also prevented the toxic action of norleucine in a manner somewhat suggestive of a product of an inhibited enzymatic reaction; however, the range of concentrations which could be tested by this method was insufficient to demonstrate effectively

TABLE V

*Effect of Isoleucine on Norleucine Toxicity*Test organism, *Escherichia coli*; incubated 14 hours at 37-38°.

DL-Norleucine	DL-Methionine	Galvanometer readings	
		Without isoleucine	With DL-isoleucine (200 $\gamma$ per 10 cc.)
$\gamma$ per 10 cc.	$\gamma$ per 10 cc.		
0	0	57	55
10	0	1	50
30	0		2
0	10	54	58
100	10	55	57
300	10	17	56
1000	10	2	15
3000	10		7
0	30	50	53
300	30	56	53
1000	30	2	53
3000	30		3
Antibacterial index.....		30 Ca.	100 Ca.

TABLE VI

*Relative Effect of Leucine, Isoleucine, and Valine on Toxicity of Norleucine*Test organism, *Escherichia coli*; incubated 15 hours at 38-39°.

Amino acids*		Galvanometer readings					
		DL-Norleucine, $\gamma$ per 10 ml.					
		0	300	1000	3000	10,000	30,000
	$\gamma$ per 10 cc.						
None		50	15	1			
DL-Valine	200	50	45	6	1		
"	2000	48	46	20	1		
DL-Isoleucine	200	50	40	29	1		
"	2000	49	40	23	1		
DL-Leucine	200	49	50	33	1		
DL-Leucine DL-Isoleucine DL-Valine	200 each	51	50	47	19	2	
DL-Isoleucine DL-Valine	200 "	49	48	44	1		
DL-Isoleucine DL-Valine	2000 "	48	48	47	43	9	1

\* In the presence of 10  $\gamma$  per 10 cc. of DL-methionine.



that this was the case. Neither isoleucine nor valine at concentrations of 1 mg. per 10 cc. had any appreciable effect on the toxicity of norleucine for *Lactobacillus pentosus* in this medium.

In Table VIII, the effect of leucine and glutamic acid and the two combined on the toxicity of norleucine for *Escherichia coli* is indicated.

TABLE VII

*Effect of Methionine and Leucine on Toxicity of Norleucine for Lactobacillus pentosus*

Test organism, *Lactobacillus pentosus*; incubated 40 hours at 30°.

DL-Norleucine	Galvanometer readings	
	DL-Methionine	DL-Leucine
	0 $\gamma$ per 10 cc.	10 $\gamma$ per 10 cc.
0	47	52
300	44	46
1,000	14	15
3,000	3	4
	1 $\gamma$ per 10 cc.	30 $\gamma$ per 10 cc.
0	53	48
300	48	56
1,000	24	27
3,000	6	14
10,000		2
	3 $\gamma$ per 10 cc.	100 $\gamma$ per 10 cc.
0	56	53
1,000	31	54
3,000	25	42
10,000	7	25
	10 $\gamma$ per 10 cc.	300 $\gamma$ per 10 cc.
0	60	61
3,000	53	65
10,000	38	60

Each exerted its effect independently of the other. Leucine in this case increased the antibacterial index 10-fold, glutamic acid 3-fold, and the two combined increased the antibacterial index 30-fold. The effect obtained with glutamic acid can also be obtained with  $\alpha$ -ketoglutaric acid (at concentrations as low as 10  $\gamma$  per 10 cc.) or with pantothenic acid (1  $\gamma$  per 10 cc.) or thiamine (1  $\gamma$  per 10 cc.). The effect as indicated in Table VIII is

not enhanced by increased concentrations of these substances, as is the case with leucine and with a mixture of isoleucine and valine.

Separate experiments with *Escherichia coli* indicated that DL-threonine (100  $\gamma$  per 10 cc.) and DL-homocystine (100  $\gamma$  per 10 cc.) were capable of preventing the toxicity of norleucine at concentrations 3 to 10 times that

TABLE VIII

*Effect of Leucine and Glutamic Acid on Toxicity of Norleucine*

Test organism, *Escherichia coli*; incubated 15 hours at 37–38°.

DL-Norleucine	DL-Methionine	Galvanometer readings, supplement			
		None	DL-Leucine (600 $\gamma$ per 10 cc.)	L-Glutamic acid (300 $\gamma$ per 10 cc.)	DL-Leucine (600 $\gamma$ per 10 cc.), L-glu- tamic acid (300 $\gamma$ per 10 cc.)
$\gamma$ per 10 cc.	$\gamma$ per 10 cc.				
0	1	55	56	55	56
30	1	54	55	58	55
100	1	1	50	49	57
300	1		47	1	57
1,000	1		1		32
3,000	1				2
0	3	55	55	55	55
100	3	56	55	54	57
300	3	9	54	45	57
1,000	3	1	47	11	53
3,000	3		6	2	15
10,000	3		2		4
0	10	55	55	57	57
300	10	46	56	57	59
1,000	10	4	54	35	57
3,000	10		31	6	47
10,000	10		5	2	16
30,000	10		3		4
0	30	55	54	55	55
1,000	30	21	52	54	57
3,000	30	4	48	32	55
10,000	30		14	2	43
30,000	30		4		10
Antibacterial index.....		100 Ca.	1000 Ca.	300 Ca.	3000 Ca.

necessary for the same degree of inhibition in a salts-glucose medium in the absence of these substances. However, in a medium containing 10  $\gamma$  per 10 cc. of DL-methionine, these amino acids exerted no effect.

Norleucine in separate tests was found to be synergistic with 2-oxo-4-imidazolidinecaproic acid in preventing the growth of *Escherichia coli*.

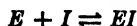
By combining each analogue in approximately one-fourth the amount necessary alone for complete inhibition of growth, the growth of *Escherichia coli* in the salts-glucose medium was completely prevented.

#### DISCUSSION

In previously reported considerations of competitive analogue-metabolite growth inhibitions of bacteria, exogenous substances other than the metabolite which are capable of preventing the toxicity of the analogue are (a) precursors of the metabolite (5), (b) the product of the enzymatic reaction "blocked" by the analogue (4), and (c) substances exerting a "sparing action" on the product of this enzymatic reaction (10, 11). A fourth type of agent capable of exerting such an effect includes (d) substances capable of increasing the effective enzyme concentration.

The effect of a precursor (a) of the metabolite is "diluted" (5) by increasing the concentration of the metabolite, and the antibacterial index determined at the higher concentration of the metabolite is identical with that determined in the absence of the precursor. Upon supplying adequate amounts for growth of the product of the "blocked" enzymatic reaction (b), the analogue either becomes ineffective as a growth inhibitor or at higher concentrations affects another enzyme system utilizing the metabolite (4). In the latter case, the antibacterial index corresponding to this new system is higher than that determined in the absence of the product. The antibacterial index determined in the presence of reversing agents of types (c) (10, 11) and (d) invariably is increased over that determined in the absence of such agents.

The actions of reversing agents of types (c) and (d) are demonstrated most readily by the following simple system under testing conditions which have limitations previously described (4).



From these equations, as previously discussed (4),

$$\begin{aligned} [I] &= K_I [EI] \\ [S] &= K_S [ES] \end{aligned} \quad (1)$$

where  $K_I$  and  $K_S$  are the dissociation constants of the inhibitor (analogue)-enzyme complex and the substrate (metabolite)-enzyme complex, respectively,  $[I]$  and  $[S]$  are the concentrations of the inhibitor and substrate, respectively, and  $[EI]$  and  $[ES]$  are the concentrations of the inhibitor-enzyme complex and the substrate-enzyme complex.

If  $[E_t]$  represents the total effective enzyme concentration, then

$$[E_t] = [ES] + [EI] + [E] \quad (2)$$

where  $[E]$  represents the concentration of free enzyme not associated with either the substrate or inhibitor. Since under testing conditions,  $[E] \rightarrow 0$  with increases in the concentration of substrate and inhibitor, *i.e.* enzyme saturation is practically attained,  $[E]$  may be neglected in determining  $[EI]$ , which is relatively large. Thus when  $[E]$  is neglected in equation (2),

$$[EI] = [E_i] - [ES] \quad (3)$$

Substituting for  $[EI]$  in equation (1) gives

$$\frac{[I]}{[S]} = \frac{K_i ([E_i] - [ES])}{K_s [ES]} \quad (4)$$

Since the ratio of  $[I]:[S]$  is determined under conditions such that the rate of synthesis of the product,  $P$ , from  $ES$  must be constant (4), under the defined condition in which only  $[I]$ ,  $[S]$ , and  $[E_i]$  may be varied by exogenous substances,  $[ES]$  must be constant and relatively small in comparison to  $[E_i]$ . Consequently the antibacterial index,  $[I]:[S]$ , will vary almost directly with  $[E_i]$ .

Thus, substances capable of increasing the effective concentration of the inhibited enzyme ( $d$ ) will prevent the toxicity of the analogue inhibitor in such a manner as to increase the antibacterial index.

The same line of reasoning can be applied to substances exerting a "sparing action." If exogenous substances act in such a manner as to decrease the amount of product  $P$  necessary for growth,  $[ES]$  must be diminished by increasing  $[I]:[S]$  if growth of the organism is to be prevented. In this case it is apparent from equation (4) that if  $[E_i]$  is assumed to be constant in the absence of reversing agents of type ( $d$ ), the antibacterial index varies almost inversely with  $[ES]$ , since  $[E_i]$  is relatively large in comparison with  $[ES]$  under testing conditions. Since the amount of product synthesized is directly proportional to  $[ES]$ , the decrease in the amount of  $P$  required by the organism resulting from the addition of these substances exerting a "sparing action" is reflected by practically a proportional increase in the antibacterial index.

Since reversing agents of types ( $b$ ), ( $c$ ), and ( $d$ ) are capable of effecting an increase in the antibacterial index, a system of differentiating these effects is desirable. Although, it has not as yet been possible to devise a system for isolated cases, a group of agents effecting changes in the antibacterial index can usually be differentiated into the various classes.

Reversing agents of type ( $c$ ) and ( $d$ ) exert their effects independently of the other, but neither exerts an effect in the presence of the product type ( $b$ ). Usually the change in the antibacterial index resulting from supplying a reversing agent of type ( $d$ ) will be of a low order of magnitude, usually not detected under testing conditions, while that of type ( $b$ ) is usually relatively large. When the "sparing action" of a reversing agent

of type (c) is that of supplying a secondary product derived from the primary product, the magnitude of the change in the antibacterial index is often relatively large. Other types of sparing actions are less frequently encountered.

Hence, for the great majority of cases, on supplying a substance to the growth medium, an increase in the antibacterial index of 3-fold or greater is a good indication that the metabolite functions directly or indirectly in the biosynthesis of the substance or its equivalent.

A study of the competitive inhibition by norleucine of the functioning of methionine led to the discovery of nine different substances which are reversing agents of the inhibition. The effects of two of these, threonine and homocystine, were "diluted" at higher concentrations of the metabolite and may be considered as precursors of methionine. The antibacterial indices determined in the presence of the other seven even at higher concentrations of methionine were increased over that determined in their absence. Classification of these substances under the reversing agents of types (b), (c), and (d) was desirable.

The members of one group,  $\alpha$ -ketoglutaric acid, glutamic acid, pantothenic acid, and thiamine, were interchangeable under the conditions of testing. Each member of this group of reversing agents was incapable of preventing the toxic action of norleucine beyond a definite ratio of analogue to metabolite, and in the presence of one, the others were not effective. This ratio was usually about 3 times the antibacterial index determined in their absence.

Leucine, isoleucine, and valine were a second group of factors which appeared to be slightly different in their mode of preventing the toxic action of the inhibitor. Increasing concentrations of leucine required increasing concentrations of norleucine for inhibition of growth, but the action was not strictly competitive. Either isoleucine or valine was capable of exerting an effect of a magnitude similar to that of the first group; however, a mixture of these two amino acids replaced leucine in giving this effect. The reported mutual antagonistic action of the two keto acids corresponding to valine and isoleucine (12, 13) as well as the amino acids themselves (14) suggests a basis for the requirement of a mixture of these two amino acids in order to attain the maximum effect of leucine. The effect of high concentrations of leucine was not effectively "diluted" by increased concentrations of methionine; so the effect is not merely increasing the production of methionine. A member of either the first or second group of reversing agents was capable of exerting its effect in the absence or in the presence of a member of the other group, as is demonstrated in Table VIII.

Since both thiamine and pantothenic acid function in the biosynthesis of  $\alpha$ -ketoglutaric acid, it appears that their effects may be to enhance produc-

tion of  $\alpha$ -ketoglutaric acid. 2-Oxo-4-imidazolidinecaproic acid, which apparently prevents the biosynthesis of  $\alpha$ -ketoglutarate by the organism (15), acts synergistically with norleucine in preventing growth of *Escherichia coli*. It appears that  $\alpha$ -ketoglutarate may therefore function as a reversing agent of type (d).

Since the first group shows an effect in the presence of the second group of reversing agents, the second group probably represents reversing agents of type (c). The effect of high concentrations of leucine may be due to a reverse reaction whereby leucine further "spares" the immediate product of the blocked reaction. The inactivity of the corresponding keto acids of leucine and isoleucine suggests that norleucine prevents the function of methionine in an enzyme system dealing with the transfer of amino nitrogen. The involvement of  $\alpha$ -ketoglutarate is suggestive of transamination. The activities of leucine, isoleucine, and valine indicate a common source of interchangeable amino nitrogen in their biosyntheses.

#### SUMMARY

The types of reversing agents other than the metabolite in competitive analogue-metabolite growth inhibitions are listed, and the types of effects obtained with each are discussed.

A study of the competitive inhibition of the functioning of methionine by norleucine has been made with *Escherichia coli* and *Lactobacillus pentosus*. For *Escherichia coli*, nine substances other than methionine were found to affect the inhibition. Threonine and homocystine were found to affect the inhibition in a manner characteristic of precursors. Pantothenic acid, thiamine,  $\alpha$ -ketoglutaric acid, or glutamic acid was found to be interchangeable in exerting an effect on the inhibition which was characteristic of substances increasing the effective enzyme concentration of the reaction "blocked" by norleucine. Leucine or a mixture of isoleucine and valine exerted an effect which suggests that methionine functions in the biosynthesis of these amino acids, probably in the amination, since the corresponding keto acids were inactive.

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## LETTERS TO THE EDITORS

### PHOSPHORYLATION COUPLED TO ELECTRON TRANSPORT BETWEEN DIHYDRODIPHOSPHOPYRIDINE NUCLEOTIDE AND OXYGEN\*

Sirs:

Measurement of efficiency of aerobic phosphorylation coupled to oxidation of pyruvate over the Krebs tricarboxylic acid cycle has disclosed that an average of 3 moles of inorganic phosphate becomes esterified during the passage of a pair of electrons from substrate to oxygen.<sup>1</sup>

#### *Phosphorylation Coupled to DPNH<sub>2</sub> Oxidation*

\* Warburg vessels contained 0.30 ml. of enzyme preparation, 0.005 M MgSO<sub>4</sub>, 0.05 M KCl, 0.02 M glycylglycine buffer, pH 7.4, 0.002 M ATP,  $1.5 \times 10^{-6}$  M cytochrome c, 486,000 counts per minute, P<sup>32</sup> as inorganic phosphate, and pyridine nucleotides in total volume of 1.0 ml. Incubated in air at 30° for 7 minutes. Other conditions are indicated below.

Experiment No.		Per cent of counts in acid-soluble esterified P	
		Plus DPNH <sub>2</sub> (5 mg.)	Plus DPN (5 mg.)
1	Complete system .....	28.3	1.99
2	" " ATP omitted .....	9.25	0.526
3	" " cytochrome c omitted .....	1.10	1.51
4	" " Mg <sup>++</sup> omitted .....	1.35	0.296
5	" " (anaerobic) .....	2.42	
6	" " + 0.018 M NaF .....	37.0	
7	" " + 0.009 " arsenite .....	1.68	
		No pyridine nucleotide	
8	Zero time .....	0.047	
9	Complete system .....	4.65	
10	" " + 0.01 M malate .....	33.8	

To determine whether esterification of inorganic phosphate is coupled to the passage of electrons from reduced diphosphopyridine nucleotide (DPNH<sub>2</sub>) to oxygen, we have used P<sup>32</sup> as a tracer. DPNH<sub>2</sub> of 70 per cent

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<sup>1</sup> Ochoa, S., *J. Biol. Chem.*, **151**, 493 (1943).



purity<sup>2</sup> was incubated aerobically with a particulate fraction of rat liver which catalyzes all the reactions of the Krebs cycle and fatty acid oxidation.<sup>3</sup> The medium contained inorganic P labeled with  $P^{32}$ ,  $Mg^{++}$ , cytochrome *c*, and ATP. The reaction was stopped with trichloroacetic acid and the acid-soluble esterified P fraction then separated for determination of radioactivity. As a control, oxidized DPN of 56 per cent purity was substituted for  $DPNH_2$ . The table shows the results of this experiment and the effects of the omission of components, anaerobiosis, and the presence of inhibitors.

It can be seen that a large incorporation of inorganic  $P^{32}$  into the esterified fraction occurred during oxidation of  $DPNH_2$  comparable to that found when a Krebs cycle intermediate, *l*-malate, was oxidized by the system. However, when DPN was substituted for  $DPNH_2$ , no significant esterification took place. The presence of ATP,  $Mg^{++}$ , and cytochrome *c* was required for the phosphorylation which did not occur anaerobically. Fluoride increased the amount of  $P^{32}$  incorporated, whereas arsenite completely abolished the effect.

Comparable experiments with the DPN-linked oxidation of *l*- $\beta$ -hydroxybutyrate to acetoacetate in which oxygen uptakes were measured accurately corroborated the above findings and disclosed that the ability to esterify phosphate is lost after aging the enzyme at 2° for 2 days without impairing the oxidation.

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<sup>2</sup> Ohlmeyer, P., *Biochem. Z.*, **297**, 66 (1938).

<sup>3</sup> Lehninger, A. L., and Kennedy, E. P., *J. Biol. Chem.*, in press.

<sup>4</sup> National Institute of Health Research Fellow.

## A NEW BLOOD CLOTTING INHIBITOR

*Sirs:*

On attempting to purify thromboplastin, various investigators<sup>1</sup> have obtained materials which possess decreased thromboplastic activity. In our work we have confirmed these observations.<sup>2</sup> The question arises as to what causes this decrease in thromboplastic activity. Is it that a component inherent to the thromboplastin molecule has been denatured, inactivated, or removed, thus inactivating the molecule? Or is it due to the presence of an inhibitor, the concentration of which has been increased by the attempted purification procedure, thus causing a decrease in the observed thromboplastin activity? It is our belief that the reduction in thromboplastic activity is due to an increase in concentration of the thromboplastin-inhibitor. Chemical isolation and identification of such a substance might indirectly point to the chemical structure of thromboplastin, since it is a known fact that most biologically active compounds have a structure similar to their inhibitors.

We have investigated the possibility of isolating a thromboplastin inhibitor. By chemical fractionation, a material possessing inhibitory properties was obtained from the phosphatide fraction of dried beef brain and rabbit lung thromboplastins, and also from soy bean phosphatides and human plasma.<sup>3</sup> In our preliminary work our most active inhibitory fraction had chemical properties similar to inositol phosphatides isolated from brain and soy beans. On chemical analysis, the following data were obtained: brain inositol phosphatide, C 53.3, P 4.3, N 1.0, ash 17.1; soy bean inositol phosphatide, C 54.1, P 2.9, N 1.1, ash 13.2. The analytical data compare favorably with the analysis given by Folch<sup>4</sup> on brain inositol phosphatide, and by Woolley<sup>5</sup> on soy bean inositol phosphatide. To verify the identity of our thromboplastin inhibitor preparation, we then isolated brain and soy bean inositol phosphatides by the methods of Folch<sup>4</sup> and Woolley.<sup>5</sup> The material isolated in this manner had the same properties as our thromboplastin inhibitory fraction, indicating that it is identical with the inositol phosphatide fraction. Samples of inositol phosphatide

<sup>1</sup> Chargaff, E., in Nord, F. F., and Werkman, C. H., *Advances in enzymology and related subjects*, New York, 5, 31 (1945). Quick, A. J., *The hemorrhagic diseases*, Chicago (1942). Studer, A., *Volume jubilaire en l'honneur de M. Emile Christophe Varell*, Bale (1946).

<sup>2</sup> Overman, R. S., and Wright, I. S., unpublished data.

<sup>3</sup> Overman, R. S., *Conference on blood clotting and allied problems*, Josiah Macy, Jr., Foundation, New York, Feb. 16-17 (1948).

<sup>4</sup> Folch, J., *J. Biol. Chem.*, **146**, 35 (1942).

<sup>5</sup> Woolley, D. W., *J. Biol. Chem.*, **147**, 581 (1943).

isolated from brain and soy beans were generously supplied by Dr. H. E. Carter of the University of Illinois. These samples also possessed inhibitory activity similar to that of our thromboplastin inhibitory preparation. At present we are working on the determination of the chemical structure of this inhibitor and its biological action on animals.

When rabbit lung thromboplastin is used, 0.5 mg. of our most active preparation prolongs the prothrombin time (Quick method) from 19 to 100 seconds on normal human plasma. 1 mg. of the inhibitor prolongs the prothrombin time from 19 to 200 seconds. By the use of whole blood and the determination of the coagulation time by the Lee-White method, 2.0 mg. of our preparation prolong the clotting time from 6 to 50 minutes. 5.0 mg. of the inhibitor prolong the coagulation time from 6 minutes to over 72 hours.

According to Carter,<sup>6</sup> lipositol (inositol phosphatides) inhibits the antibiotic activity of streptomycin. Logically this would seem to indicate that streptomycin might have a structure similar to that component of thromboplastin which is inhibited by lipositol. We have found that streptomycin hydrochloride (Merck) and streptomycin sulfate (Eli Lilly and Company) have an effect similar to inositol phosphatide in that they prolong the prothrombin time and coagulation time of blood. Further work is in progress to ascertain the mechanism of this action.

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<sup>6</sup> Rhymer, I., Wallace, G. I., Byers, L. W., and Carter, H. E., *J. Biol. Chem.*, **169**, 457 (1947).

# INHIBITION OF THE PHOSPHORYLATION OF GLUCOSE BY MENINGOCOCCAL ENDOTOXIN\*

*Sirs:*

In the course of investigations dealing with the mode of action of bacterial endotoxins, it was found that meningococcal endotoxin, prepared in Dr. C. P. Miller's laboratory according to a method described elsewhere,<sup>1</sup> inhibited the *in vitro* utilization of glucose by rat muscle extracts. This inhibition did not occur if Zn-free insulin<sup>2</sup> was added to the reaction mixture. The preparation of the rat muscle extracts and the determina-

*Inhibition of Glucose Utilization by 2 Mg. of Meningococcal Endotoxin*

Series No.	Normal	Plus 2 mg.* men- ingococcal endotoxin	2 mg.* meningococ- cal endotoxin + 100 $\gamma$ insulin	Average per cent inhibition
1	535	350		28
	480	380		
	517	375		
2	610	400		36
	580	392		
	598	360		
3	480	310		35
	500	340		
	470	295		
4	590	480	560	Without insulin 24; with insu- lin 0
	610	360	640	
	570	440	650	
	550	490	540	

\* In terms of solid content.

tion of glucose utilization were carried out under the experimental conditions described by Colowick, Cori, and Slein.<sup>3</sup> The muscle extracts were kept in an ice box for 2 hours previous to the experiment in order to obtain maximal enzyme activity. The results expressed as micrograms of glucose utilized per reaction mixture are summarized in the table.

Since under these circumstances the glucose utilization can be considered a measure of hexokinase activity, it was concluded that meningococcal

\* This investigation was supported jointly by the United States Navy Office of Naval Research and the University of Chicago.

<sup>1</sup> Kun, E., *Proc. Soc. Exp. Biol. and Med.*, **66**, 197 (1947).

<sup>2</sup> The amorphous preparation of Zn-free insulin was kindly supplied by Dr. Chen of Eli Lilly and Company.

<sup>3</sup> Colowick, S. P., Cori, G. T., and Slein, M. W., *J. Biol. Chem.*, **168**, 583 (1947).

endotoxin inhibits this enzyme. Further studies with purified hexokinase are in progress.

These experiments strongly suggest that the disturbances of the carbohydrate metabolism in meningococcal toxemic rabbits<sup>4</sup> and in meningococcal meningitis in humans reported by Fox, Kuzma, and Washman<sup>5</sup> can be explained by a direct effect of meningococcal endotoxin on enzymatic reactions in the host.

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<sup>4</sup> Kun, E., and Miller, C. P., *Proc. Soc. Exp. Biol. and Med.*, in press.

<sup>5</sup> Fox, M. J., Kuzma, F. J., and Washman, W. T., *Arch. Int. Med.*, **79**, 614 (1947).

# ANTIPERNICIOUS ANEMIA EXTRACTS AND TYROSINE METABOLISM IN THE SCORBUTIC GUINEA PIG

Sirs:

Swendseid, Burton, and Bethel<sup>1</sup> have shown that the pernicious anemia patient in relapse excretes large quantities of keto acid and hydroxyphenyl compounds (calculated as tyrosine). With the administration of appropriate liver extracts the level of excretion of these metabolites returned to the normal value simultaneously with the characteristic improvement in the blood picture. Since the scorbutic but not the normal guinea pig

Liver extract injected	Keto acid excretion before and after injection		
	24 hrs. before	24 hrs. after	Per cent decrease per unit
<i>units</i>	<i>mg.</i>	<i>mg.</i>	
15	202	159	1.42
20	237	172	1.37
20	195	135	1.54
20	215	186	0.67
Average.....			1.25 ± 0.29
7.5	244	199	2.46
7.5	314	265	2.08
15	253	208	1.19
22.5	325	156	2.31
Average.....			2.01 ± 0.41

exhibits the excretion of tyrosine metabolites when extra tyrosine is fed,<sup>2</sup> it is of interest to determine the effect of similar liver extracts. In preliminary experiments<sup>3</sup> in 1943 very striking positive results were obtained. More recently, additional detailed experiments have been performed. Scorbutic guinea pigs receiving 200 mg. of extra tyrosine per 100 gm. of body weight per day showed keto acid excretion values, as illustrated in the accompanying table. As also may be seen, the subcutaneous injection of antipernicious anemia preparations caused a marked reduction in the amount of keto acid excretion. Total phenolic (tyrosyl) values paralleled the keto acid values. With a given preparation the effect observed may

<sup>1</sup> Swendseid, M. E., Burton, I. F., and Bethel, F. H., *Proc. Soc. Exp. Biol. and Med.*, **52**, 202 (1943).

<sup>2</sup> Basinski, D. H., and Sealock, R. R., *J. Biol. Chem.*, **166**, 7 (1946).

<sup>3</sup> Sealock and Basinski, unpublished.

be roughly correlated with the number of units injected. Other liver extracts yielded similar results, although correlation between extracts on the basis of unitage was relatively poor. Since the unitage of such preparations may be uncertain, we have continued an investigation of the procedure as an experimental and quantitative assay method for either the antipernicious anemia factor or other factors present and responsible for the observed effect. Under any circumstances, the extracts produce an effect on tyrosine metabolism similar in gross aspects to the effect of ascorbic acid and the more recently announced effect of pteroylglutamic acid.<sup>4</sup> However, they do not, as we have also observed, increase the *in vitro* oxidation of tyrosine by liver tissue, as do ascorbic acid<sup>5</sup> and pteroylglutamic acid.<sup>6</sup>

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<sup>4</sup> Woodruff, C. W., and Darby, W. J., *J. Biol. Chem.*, **172**, 851 (1948).

<sup>5</sup> Lan, T. H., and Sealock, R. R., *J. Biol. Chem.*, **155**, 483 (1944).

<sup>6</sup> Rodney, G., Swendseid, M. E., and Swanson, A. L., *Federation Proc.*, **6**, 419 (1947).

# PYRIMIDINE DERIVATIVES AS ANTAGONISTS OF PTEROYLGLUTAMIC ACID

Sirs:

Pteroylglutamic acid (PGA) has been assigned the rôle of the prosthetic group of an enzyme concerned with the synthesis of thymine<sup>1</sup> or thymine and purine<sup>2</sup> or their equivalents. In studies of a large number of pyrimidine derivatives, we have found an inhibition of the growth of *Lactobacillus casei* with PGA in the absence of purine to be a property of nearly all 2,4-diaminopyrimidines and their condensed systems, most 2-aminopyrimidine

Compound	Antagonist added	PGA	Adenine	Titer
	$\gamma$ per ml.	mg per ml.	$\gamma$ per ml.	ml. 0.1 n per 10 ml.
2,6-Diaminopurine	0	0.045	0	6.65
	0.1	0.045	0	2.20
	5.0	0.045	0	0.85
	0	0.045	1.0	6.90
	5.0	0.045	1.0	6.90
	0	2.8	0	15.10
	0.1	2.8	0	14.50
	0.5	2.8	0	6.55
2,4-Diamino-6,7-dimethylpteridine	0	0.045	0	7.35
	0.5	0.045	0	5.75
	5.0	0.045	0	2.95
	0	0.045	10	8.30
	0.5	0.045	10	7.75
	5.0	0.045	10	5.30
	0	2.8	0	15.10
	12.5	2.8	0	15.10

derivatives, and several 2-amino-4-hydroxypyrimidine systems, including pteridines. These compounds demonstrably become involved in several enzyme systems, as can be shown by studies of (1) the reversibility of the inhibition by PGA, (2) the reversibility by purines, and (3) the effects on growth with thymine. These studies appear to segregate several independently variable properties which vary in prominence from compound to compound but are possessed in some degree by all. For example, the inhibitions of both 2,4-diamino-6,7-dimethylpteridine and 2,6-diaminopurine can be shown to be reversed competitively by PGA or by purines under chosen conditions, but they differ in the proportionate amounts of the two metabolites required to reverse a given inhibition and the range of

<sup>1</sup> Stokes, J. L., *J. Bact.*, **48**, 201 (1944).

<sup>2</sup> Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, **170**, 133 (1947).



concentrations over which the reversals can be demonstrated. The action of the pteridine is reversible by PGA over a wide range of concentrations and by purines over a relatively restricted range, but with 2,6-diaminopurine the reversal by purines covers a much greater range of concentrations of antimetabolite than that by PGA (see the table). The 2-amino-4-hydroxypteridine derivatives, 2-aminopurine, and the 2-amino- and 2,4-diamino-6,7-dihydroxypteridines similarly produce inhibitions which are readily reversed by purines. However, these compounds cannot be considered simply as antipurines, for they interfere minimally or not at all with the utilization of exogenous purine when the microorganisms are grown with thymine rather than with PGA. On the other hand, the diaminodimethylpteridine, which has been regarded primarily as a PGA antagonist,<sup>3</sup> has a considerable inhibitory effect on *L. casei* when the organism is grown with thymine and adenine. In this respect and in the reversals of the antagonism of PGA, 2,4-diamino-5-methyl- and 5,6-dimethylpyrimidines are intermediate in behavior between the diaminodimethylpteridine and the diaminopurine.

The diaminopurine can be shown to be involved in at least two systems concerned with purine, for its effects are reversible equally well by adenine or guanine at low concentrations, but by adenine and not by guanine at higher concentrations. In view of the multiplicity of effects which these studies reveal, we feel that a certain amount of restraint in interpretation is called for. To regard any of these compounds as exhibiting a simple metabolite-antimetabolite relationship for a single enzyme system is a simplification which becomes acceptable only when other effects have been ruled out.

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<sup>3</sup> Daniel, L. J., Norris, L. C., Scott, M. L., and Heuser, G. F., *J. Biol. Chem.*, **169**, 689 (1947).

# OBSERVATION ON THE UTILIZATION OF GLYCINE IN THE BIOSYNTHESIS OF HEMOGLOBIN\*

*Sirs:*

In the course of experiments on porphyrin biosynthesis and degradation we have attempted to prepare tagged hemoglobin protoporphyrin with carboxyl-labeled glycine. It has been demonstrated that glycine serves as a nitrogenous precursor of protoporphyrin.<sup>1</sup> Recently it has been reported that the  $\alpha$ -carbon of glycine is also utilized in protoporphyrin synthesis.<sup>2</sup>

One 10 kilo dog, made anemic by bleeding, received by stomach tube in three portions over a period of 3 days 100 mg. of glycine<sup>3</sup> labeled with C<sup>14</sup> in the carboxyl group (total activity  $5 \times 10^7$  counts per minute). Samples of blood were obtained on the 11th, 14th, 20th, 37th, and 52nd days following administration. Crystalline protoporphyrin methyl ester<sup>4</sup> and globin<sup>5</sup>

Time	Protoporphyrin methyl ester activity	Globin activity*
<i>days</i>	<i>c.p.m. per mg.</i>	<i>c.p.m. per mg.</i>
11	<0.1	
14	<0.1	
20		$21.3 \pm 0.5$
37	<0.1	$22.8 \pm 1.5$
52	<0.1	$21.5 \pm 1.0$

\* The deviations shown are estimated from average deviations of duplicate samples.

were obtained and the radioactivity measured with a thin walled Geiger-Müller counter.<sup>6</sup> The results are given in the accompanying table. No radioactivity was observed in the protoporphyrin. The globin showed an activity which has remained constant throughout the period of observation (up to 52 days).

One rat of 100 gm. weight, kept on a protein-free diet, received by stomach tube in four portions during a period of 3 days 200  $\gamma$  of the same labeled glycine (total activity of  $2.1 \times 10^8$  counts per minute). 20 days

\* Supported by grants from Mr. John Mosby and Mr. John Greenberg.

<sup>1</sup> Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **159**, 567 (1945).

<sup>2</sup> Altman, K. I., Casarett, G. W., Masters, R. E., Noonan, T. R., and Salomon, K., *Federation Proc.*, **7**, 2 (1948).

<sup>3</sup> We are indebted to Dr. R. B. Loftfield, Massachusetts Institute of Technology, for the synthesis of the labeled glycine.

<sup>4</sup> Grinstein, M., *J. Biol. Chem.*, **167**, 515 (1947).

<sup>5</sup> Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, **13**, 469 (1930).

<sup>6</sup> Kamen, M. D., *Radioactive tracers in biology*, New York, 175 (1947).

later the animal was bled to death, crystalline protoporphyrin methyl ester was prepared from the collected blood, and its activity was measured. Again no radioactivity was observed. The remaining total protein of the cells had an activity of  $1.5 \pm 0.2$  counts per minute.

These results show that the *carboxyl carbon* of glycine is not utilized in the protoporphyrin biosynthesis, but is incorporated into the globin molecule.<sup>7</sup> Further studies are in progress.

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<sup>7</sup> The lack of utilization of glycine carboxyl in the formation of protoporphyrin has been observed independently by N. Radin, D. Rittenberg, and D. Shemin, private communication.

# THE EFFECT OF SULFONAMIDE IN THE DIET OF PREGNANT RATS ON ERYTHROPOIESIS IN THE FETUSES\*

Sirs:

We have been attempting to produce an anemia in fetal rats by various techniques. The inclusion of sulfonamides in the diet of adult rats is known to produce an anemia which may be relieved by the administration of liver concentrates or folic acid.<sup>1</sup> An experiment attempting to produce an anemia in fetal rats by feeding the pregnant animals a diet containing succinylsulfathiazole gave anomalous results.

Diet	Maternal blood		No. of litters	No. of fetal rats	Fetal blood	
	Hemo- globin	Eryth- ro- cytes			Hemoglobin*	Erythrocytes*
	gm. per cent	10 <sup>6</sup> per c.mm.			gm. per cent	10 <sup>6</sup> per c.mm.
Basal.....	10.05	5.22	7	44	12.67 (11.3-13.6)	2.82 (2.41-3.46)
“ + folic acid.....	14.70	7.91	4	34	10.60 (9.6-11.8)	2.38 (2.08-2.74)
Normal stock.....			5	45	10.40 (9.8-11.8)	2.28 (2.01-2.42)

\* The figures in parentheses represent the range of the mean values of the litters.

Two groups of female rats were kept on a basal synthetic diet containing 1 per cent succinylsulfathiazole, the crystalline B vitamins, and vitamins A, D, E, and K; the diet of the control group was supplemented with 2 mg. of folic acid<sup>2</sup> per 100 gm. of food. After 10 days, the rats were mated. Directly after birth, blood samples were obtained from the new-born and the maternal rats by heart puncture. The hemoglobin analyses and the erythrocyte counts are shown in the table.

Although the expected anemia appeared in the maternal rats deprived of folic acid, surprisingly the fetal blood contained a higher concentration of hemoglobin than was found in the blood of the control fetal rats. A

\* This work was supported by a grant from the Foundation for Vision for the study of Retrolental Fibroplasia.

<sup>1</sup> Spicer, S. S., Daft, F. S., Sebrell, W. H., and Ashburn, L. L., *Pub. Health Rep., U. S. P. H. S.*, 57, 1559 (1942). Franklin, A. L., Stokstad, E. L. R., Belt, M., and Jukes, T. H., *J. Biol. Chem.*, 169, 427 (1947).

<sup>2</sup> The folic acid used was about 85 per cent pure (Folvite powder, No. 7-7904). It was generously supplied by Dr. M. C. Lockhart of the Lederle Laboratories.

statistical analysis of the data showed that the difference between the mean values for hemoglobin is significant, while that for the erythrocyte counts is of doubtful significance.<sup>3</sup> There appear to be no significant differences between the values for the control fetal blood and those for the blood of fetuses from mothers kept on a normal stock diet.

It appears possible that the higher concentration of hemoglobin in the fetuses from the folic acid-deficient maternal rats may be due to a greater availability of hemoglobin precursors; *e.g.*, iron. The maternal rat, being unable to utilize the hemoglobin precursors normally because of the relative deprivation of folic acid, may make the precursors more readily available for the fetuses.

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<sup>3</sup> When litters were taken as the statistical unit, the significance between the mean values for the hemoglobin was found to be  $P < 0.01$ , for the erythrocyte counts  $P = 0.07$  ("Student"  $t$  test). When single fetuses were used as the statistical units, the differences were all found to be highly significant.

## INHIBITION OF XANTHINE OXIDASE AND RELATED ENZYMES BY 6-PTERIDYL ALDEHYDE\*

*Sirs:*

We have recently reported<sup>1</sup> the inhibition of xanthopterin oxidase and xanthine oxidase by synthetic pteroylglutamic acid (PGA). However, since an exceptionally pure sample<sup>2</sup> of PGA, which was later examined, possessed less than one-sixth of the inhibitory power previously observed, it appears possible that the effect is due to traces of a contaminant. The observation by Lowry and Bessey<sup>3</sup> that 2-amino-4-hydroxy-6-pteridyl aldehyde, a primary photofission product of PGA, strongly inhibits pteridine oxidase suggests that this aldehyde may be the active inhibitor in the above enzyme systems. We have now found that the 6-pteridyl aldehyde, prepared by hydrolysis of PGA in sulfurous acid,<sup>4</sup> is on a molar basis 200 to 400 times more active as an inhibitor of xanthine oxidase (i.e. 0.005 to 0.02  $\gamma$  per ml.) than the folic acid preparations previously studied.<sup>1</sup>

A number of other oxidases related to xanthine oxidase are also strongly inhibited by 6-pteridyl aldehyde. It was mentioned that Lowry found that the enzymic oxidation of 2-amino-4-hydroxypteridine to iso-xanthopterin is inhibited by minute amounts of the pteridyl aldehyde. Xanthopterin oxidase from milk and liver is likewise inhibited by small amounts of pteridyl aldehyde. This substance exerts also a strong effect on quinine oxidase from rabbit liver.<sup>5</sup> A concentration of 0.4  $\gamma$  of pteridyl aldehyde per ml. reduces the quinine oxidase activity to about 30 per cent of that of the uninhibited sample. This observation may have pharmacological interest inasmuch as human liver is reported to contain quinine oxidase.

Uricase and triose phosphate oxidase were not inhibited by the addition of pteridyl aldehyde.

The potency of 6-pteridyl aldehyde as an inhibitor is lost by addition of 2,4-dinitrophenylhydrazine which reacts with the free aldehyde group.

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<sup>1</sup> Kalckar, H. M., and Klenow, H., *J. Biol. Chem.*, **172**, 349 (1948).

<sup>2</sup> Kindly furnished by Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Company.

<sup>3</sup> Lowry, O. H., and Bessey, O. A., private communication.

<sup>4</sup> Hutchings, B. L., Stokstad, E. L. R., Mowat, J. H., Boothe, J. H., Waller, C. H., Angier, R. B., Semb, J., and Subbarow, Y., *Ann. New York Acad. Sc.*, **48**, 273 (1946).

<sup>5</sup> Knox, W. E., *J. Biol. Chem.*, **163**, 699 (1946).

The inhibitory effect of various Folvite fractions is also extinguished by addition of diphenylhydrazine, indicating that the inhibitory component of these preparations might be caused by traces of 6-pteridyl aldehyde or related compounds. Metallic zinc, which presumably reduces the pteridyl aldehyde, destroys the inhibitory potency. Irradiation of 6-pteridyl aldehyde, which is reported to yield pteridylcarboxylic acid,<sup>1</sup> likewise led to a disappearance of the inhibitory action.

Milk and rat liver contain an enzyme which converts pteridyl aldehyde into a non-inhibitory substance which exhibits a somewhat higher fluorescence than the original product. The nature of this conversion product is under investigation. We are inclined to believe that the previously reported enzymic transformation of pteroylglutamic acid<sup>2</sup> which manifests itself by a more or less complete loss in inhibitory potency towards xanthopterin oxidase must be ascribed to an enzymic transformation of the traces of 6-pteridyl aldehyde presumably present in the folic acid preparations used.

The action of 6-pteridyl aldehyde on the oxidative breakdown of purines warrants further investigations as to the possible effects of this highly active inhibitor on purine metabolism *in vivo*.

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<sup>1</sup> Kalckar, H. M., and Klenow, H., *J. Biol. Chem.*, **172**, 351 (1948).

# THE EFFECT OF DINITROPHENOL ON THE INCORPORATION OF ALANINE LABELED WITH RADIOACTIVE CARBON INTO THE PROTEINS OF SLICES OF NORMAL AND MALIGNANT RAT LIVER\*

Sirs:

Early experiments of Clowes and Krah1<sup>1</sup> indicated that dinitrophenol permits oxygen consumption and yet interferes with cell division of the sea urchin egg. Later studies of Hotchkiss<sup>2</sup> pointed to an interference of dinitrophenol with phosphate uptake in respiring yeast. The recent work of Loomis and Lipmann<sup>3</sup> has identified the property of dinitrophenol to uncouple the interlocked processes of respiration and phosphorylation.

DNP, molar concentration	Normal liver		Primary hepatoma	
	O <sub>2</sub> consumption	Alanine incorporation, c.p.m.	O <sub>2</sub> consumption	Alanine incorporation, c.p.m.
0	50, 57	30, 32	82, 78	285, 227
1 × 10 <sup>-5</sup>	50, 49	38, 31	89	276
5 × 10 <sup>-5</sup>	75	17	93, 105	172, 170
1 × 10 <sup>-4</sup>	70	8.6	101, 98	98, 94
5 × 10 <sup>-4</sup>	57, 51	0.9, 0.8	32, 19	1.2, 1.2
1 × 10 <sup>-3</sup>	53, 56	0, 0.5	19, 11	1.5, 1.4

Temperature 37°, pH 7.40, 100 per cent oxygen in the gas phase, incubation time 3½ hours. Oxygen consumption is expressed as microliters of oxygen consumed per 30 minutes per 0.1 mm BaCO<sub>3</sub>. The BaCO<sub>3</sub> is obtained by ninhydrin decarboxylation<sup>4</sup> of the amino acids present in hydrolysates of the slice proteins. The counts per minute (c.p.m.) are likewise expressed per 0.1 mm BaCO<sub>3</sub>, at 0.1 mm thickness of the BaCO<sub>3</sub> precipitate used for solid counting. Figures are given for duplicate vessels. Hepatomas were induced by feeding a diet containing *p*-dimethylaminoazobenzene.

Dinitrophenol has thus been shown to interfere with the formation of energy-rich phosphate bonds in a respiring system.

Previous studies<sup>4</sup> have demonstrated that C<sup>14</sup>-carboxyl-labeled alanine

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<sup>1</sup> Clowes, G. H. A., and Krah1, M. E., *J. Gen. Physiol.*, **20**, 145 (1936).

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is incorporated into the proteins of surviving rat liver slices during incubation of the slices in Warburg vessels in a Krebs-Ringer-phosphate medium to which is added tagged DL-alanine. Little or no incorporation occurs if the incubation is carried out in an atmosphere of nitrogen.

In the present work, it has been found that addition of dinitrophenol (DNP) to the above system inhibits the incorporation of alanine into protein in both normal and tumor tissue, while permitting oxygen consumption to continue. The suggestion emerges that synthesis of alanine peptide bonds depends on a source of energy-rich phosphate donors.

In higher concentrations ( $1 \times 10^{-3}$  and  $5 \times 10^{-4}$  M) the DNP had an inhibitory effect on respiration in the hepatoma slices, but little or none in the normal liver slices.

A higher rate of incorporation of alanine into hepatoma protein than into normal liver protein was found, under conditions of the slice experiments. This latter finding will be discussed at greater length in a future communication.

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# THE ESTIMATION OF FATTY ACIDS OF INTERMEDIATE CHAIN LENGTH BY PARTITION CHROMATOGRAPHY\*

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In 1942 Smith (1) demonstrated that the silica gel partition chromatogram, first developed by Martin and Synge (2), would separate a mixture of formic, acetic, propionic, *n*-butyric, and *n*-valeric acids in chloroform-1 per cent butanol into its component acids. In 1945 Ramsey and Patterson (3) confirmed this work and presented a partition chromatographic method for the qualitative separation and identification of formic, acetic, propionic, and butyric acids. In 1946 Elsdén (4) presented a method based on the work of Smith by which acetic, propionic, and *n*-butyric acids in biological materials can be quantitatively separated and estimated.

Elsdén's method is limited in that the butanol-chloroform-water system he employs will not separate fatty acids having 5 or more carbon atoms. In an investigation of the rôle of fatty acids in Cheddar cheese flavor, the present authors found it necessary to develop a rapid method for the quantitative estimation of formic, acetic, propionic, *n*-butyric, caproic, caprylic, and capric acids. By use of the system benzene-aqueous sulfuric acid in partition chromatograms such a method was devised and is presented here.

## *Preparation of Partition Chromatograms*

The partition chromatograms used consisted of glass tubes packed with an inert filler (Celite 545, a coarse diatomaceous filter aid made by Johns-Manville) moistened with the non-mobile phase, which was water or aqueous sulfuric acid. Various organic solvents were used as the mobile phase (developing solvent).

*Developing Solvents*—The developing solvents used were thiophene-free benzene, a mixture of Skellysolve B and thiophene-free benzene containing 2 per cent (by volume) of Skellysolve B, and two mixtures of butanol and chloroform containing 10 and 25 per cent (by volume) of butanol. These solvents will be called CB-10 and CB-25, respectively. Thiophene-free benzene was prepared by repeatedly shaking benzene with concentrated sulfuric acid at room temperature until the acid no longer acquired a color.

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The benzene was then washed with water, dried over pellets of sodium hydroxide, and filtered.

Prior to use on any chromatogram all developing solvents were saturated with the non-mobile phase of that chromatogram.

*Chromatogram Tubes*—Large chromatogram tubes, designated as macro chromatogram tubes, were made from Pyrex glass tubing having an inside diameter of 18 mm. These tubes were approximately 40 cm. in length and were constricted at one end to an inside diameter of 5 mm. A perforated porcelain disk bearing a thin pad of glass wool was lodged on the constriction and this served to support the column. At the bottom of the tube on the constricted end, a piece of Tygon tubing was used to attach a short piece of 6 mm. glass tubing as a delivery tip. A screw clamp was used to regulate the flow of the developing solvent.

Small chromatogram tubes, designated as micro chromatogram tubes, were made from Pyrex glass tubing having an inside diameter of 10 mm. These tubes were approximately 30 cm. in length, and developing solvent flow was controlled in the same manner as that of the macro chromatogram tubes. In the micro chromatogram tubes the column was supported by means of a thin pad of glass wool resting on a large glass bead lodged on the constriction of the tube.

*Packing of Chromatograms*—The benzene-aqueous sulfuric acid macro chromatograms were prepared as follows: To 40 gm. of Celite 545 were added 24 ml. of the non-mobile phase. This mixture was then rapidly mixed in a mortar with a pestle until a homogeneous powder was obtained. At this point developing solvent was added in sufficient amount to flood the mixture in the mortar completely. Unless otherwise specified, the developing solvent used in packing and developing all chromatograms hereafter described was thiophene-free benzene saturated with the non-mobile phase present in the finished chromatogram. When strongly hygroscopic solutions of sulfuric acid are used as the non-mobile phase, mixing the Celite 545 and the non-mobile phase in a mortar open to the atmosphere results in change of normality of the sulfuric acid prior to its introduction into the chromatogram. Once the column pack is covered with developing solvent saturated with a solution of sulfuric acid having the concentration desired in the non-mobile phase of the finished chromatogram, no further change in sulfuric acid normality occurs. In order to obtain a chromatogram ready for use with the desired normality, the following procedure was used routinely in the preparation of both macro and micro chromatograms having sulfuric acid solutions as their non-mobile phases. To the Celite 545 in a mortar was added the required amount of a sulfuric acid solution having a normality as much as one unit higher than the normality desired. This mixture was then mixed in the mortar for exactly 2 minutes, in which

time a homogeneous powder was always obtained. Immediately after mixing, a small sample (2 to 3 gm.) of the powder was rapidly transferred to a small tared, stoppered, weighing bottle, and the remaining Celite 545-sulfuric acid mixture was flooded with developing solvent saturated with a sulfuric acid solution having the normality desired in the non-mobile phase of the column. The weighing bottle was weighed and its sulfuric acid content determined by titration. Since the ratio of acid to Celite 545 is known, the weight of absorbed water can be calculated. It was found by this procedure that 28 to 32 *N* sulfuric acid solutions dropped from 0.3 to 0.5 normality unit during the 2 minute mixing period, 32 to 34 *N* solutions dropped from 0.5 to 0.8 normality unit, and 34 to 36 *N* solutions dropped from 0.8 to 1.2 normality units. Since this drop in normality varies not only with the time and intensity of mixing but also with the amount of moisture in the atmosphere, it was necessary to check final sulfuric acid normalities routinely in both macro and micro chromatograms by the procedure described above. Subsequently an alternative procedure was found by which the normality of the sulfuric acid as the non-mobile phase of the chromatogram could be checked. This alternative procedure consisted of testing the separatory power of benzene-sulfuric acid chromatograms on known mixtures of pure fatty acids.

Macro chromatogram tubes were packed as follows: The screw clamp at the bottom of the tube was closed, and a small volume of developing solvent was poured carefully down the side of the tube. This layer of liquid in the bottom of the chromatogram tube prevented disturbance of the glass wool pad by the initial addition of the packing slurry. This slurry, consisting of Celite 545, non-mobile phase, and developing solvent, was added in about ten approximately equal portions, with mechanical packing after each addition. After the initial addition and prior to mechanical packing, the screw clamp at the bottom of the tube was opened, and the developing solvent flow was regulated as desired throughout the packing operation. Mechanical packing was accomplished by means of a glass rod flattened at one end until the diameter of the flattened part was slightly less than the inside diameter of the chromatogram tube. While in use the packing rod was continually rotated to prevent the formation of vertical channels in the column pack. Care was taken to prevent packing the column so tightly that the flow rate desired during column development could not be obtained. Mechanical packing as described above continued until the height of the finished column was exactly 15 cm. above the supporting glass wool pad, with the top surface as level as possible.

The preparation and packing of benzene-aqueous sulfuric acid micro chromatograms was identical with that for the macro chromatograms with the following exceptions. Approximately one-eighth the amount of pack-

ing slurry used in packing a macro chromatogram was required to pack a micro chromatogram. The final height of column above the glass wool pad was exactly 6 cm.

During the packing and when not in use, the surfaces of all macro and micro chromatogram columns were kept covered with developing solvent. If sulfuric acid columns are allowed to stand more than a week, some sulfonation of the benzene may occur, increasing the blank titration of the effluent.

### *Chromatographic Procedure*

*Column Development*—The chromatogram was drained of developing solvent until the point was just reached at which no liquid was apparent above the top surface of the column. The screw clamp was then closed and a suitable small volume of the sample to be analyzed was carefully pipetted onto the top of the column. The screw clamp was then partially opened and, starting at this point and continuing throughout the development of the column, small equal aliquots of the developing solvent were collected in calibrated test-tubes. In micro chromatogram analyses 1 ml. aliquots of developing solvent were collected; in macro chromatograms the aliquots were 5 ml. in volume. The rate of developing solvent flow for both micro and macro chromatograms was maintained throughout development of the columns at 1 ml. per minute by means of the screw clamp. After the fatty acid sample to be analyzed had just completely passed into the column, a 0.5 ml. aliquot of developing solvent was carefully added to the top of the column to rinse remaining traces of the sample into the column. After the rinse had just completely passed into the column, development of the chromatogram was carried out in the normal manner.

*Analyses*—Effectiveness of separation by the chromatogram of the fatty acids in the sample analyzed was determined by titration of the developing solvent aliquots removed at the bottom of the chromatogram tube. To each aliquot was added twice its volume of neutral 95 per cent ethanol. These solutions were then titrated to the phenol red end-point with 0.1 N aqueous barium hydroxide from a burette calibrated at 0.01 ml. intervals.

When chromatograms, having sulfuric acid solutions as their non-mobile phases, were used to separate known mixtures of fatty acids, it was found that recoveries of individual fatty acids as measured by direct titration were always high (105 to 160 per cent). This was due to a carry over of sulfuric acid in the developing solvent in the presence of fatty acid. It was found that the sulfuric acid present in the developing solvent from macro chromatograms could be completely removed, without change in the fatty acid content, by passage of the developing solvent through a glass delivery tube 6 mm. in diameter and 10 cm. long, packed with 0.5 gm. of

non-absorbent cotton. A similar cotton filter for micro chromatograms was made by using a piece of 4 mm. glass tubing packed with 0.2 gm. of cotton. The sulfuric acid absorption capacities of the 0.5 and 0.2 gm. cotton filters are 0.6 and 0.2 milliequivalents respectively. Since the passage of 1 milliequivalent of fatty acid through a benzene-sulfuric acid (28 to 36 N) chromatogram causes the carry over of approximately 0.5 milliequivalent of sulfuric acid in the developing solvent, the total fatty acid content of the samples analyzed in sulfuric acid macro and micro chromatograms was kept less than 0.8 and 0.3 milliequivalents respectively. Even though it was possible with many fatty acid samples to make two or three analyses with the same cotton filter, routinely a fresh cotton filter was used for each column developed.

#### *Behavior of Fatty Acids in Benzene-Aqueous Sulfuric Acid System*

*Benzene-Water System*—In any partition chromatogram only compounds having differing partition coefficients between the two immiscible phases can be separated. In the butanol-chloroform-water system the partition coefficients of the straight chain fatty acids having 4 carbon atoms or less differ sufficiently so that their quantitative separation and estimation are easily effected by the use of a silica gel partition chromatogram by this system (4). The same quantitative separation and estimation of *n*-butyric, propionic, acetic, and formic acids were found to be possible with a partition chromatogram prepared as previously described, but with water as the non-mobile phase and benzene as the developing solvent. With such a chromatogram, it is obvious that formic, acetic, and propionic acids can be quantitatively separated and estimated in the presence of any number of saturated fatty acids having 4 or more carbon atoms.

In Fig. 1 the results of a typical separation of formic, acetic, propionic, and *n*-butyric acids on a benzene-water-Celite 545 micro chromatogram are presented. The fatty acid sample used was a 1 ml. sample of CB-10 containing 100 micromoles of each of the four fatty acids. As may be seen, *n*-butyric, propionic, and acetic acids passed through the column in that order in the first 90 ml. of developing solvent and were completely separated. Formic acid remained fixed on the column and did not appear during the passage of 200 ml. of developing solvent through the chromatogram. Recoveries of *n*-butyric acid and propionic acid were good, being 98.2 and 97.3 per cent respectively. Acetic acid recovery was high (115.5 per cent) because of the wide dilute solute zone it formed during its slow passage of the column. As a result formic acid recovery, calculated by difference, was low (89.0 per cent).

By changing the mobile phase during the development of the water-Celite 545 chromatogram, it was found that acetic acid recovery was im-

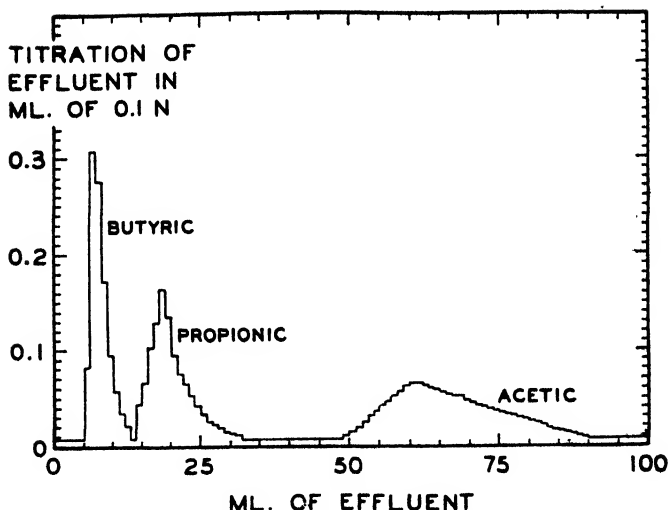


FIG. 1. Separation of *n*-butyric, propionic, acetic, and formic acids on a benzene-water-Celite 545 partition micro chromatogram. Titrations were made of 1 ml. aliquots of developing solvent from the base of the column. Recoveries of *n*-butyric, propionic, and acetic acids were 98.2, 97.3, and 115.5 per cent respectively. Formic acid remained fixed on the column and its recovery by difference was 89.0 per cent.

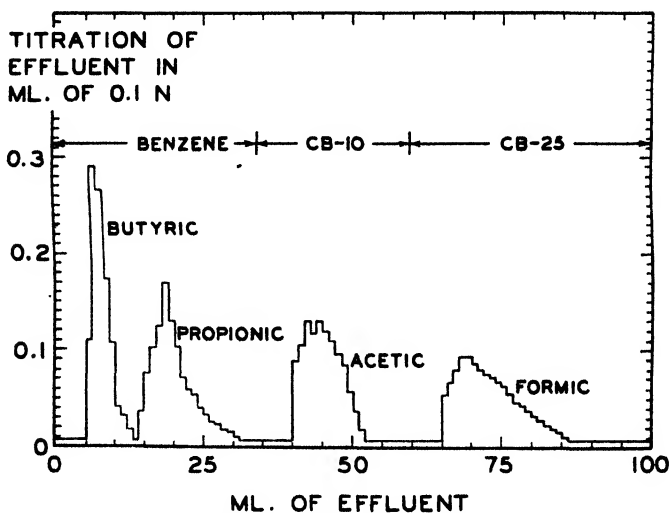


FIG. 2. Separation of *n*-butyric, propionic, acetic, and formic acids on a water-Celite 545 partition micro chromatogram with three developing solvents. Titrations were made of 1 ml. aliquots of developing solvent from the base of the column. Recoveries of *n*-butyric, propionic, acetic, and formic acids were 98.6, 97.6, 100.9, and 102.5 per cent respectively.

proved and formic acid could be determined directly by titration. Fig. 2 shows the results obtained when the experiment described in Fig. 1 was repeated with three developing solvents. As may be seen, after all the *n*-butyric and propionic acids had been removed by the passage of benzene through the column (30 to 40 ml.), CB-10 was substituted for the benzene and column development was continued. This developing solvent narrowed the acetic acid zone and rapidly eluted it from the column. After all the acetic acid had been removed, CB-10 was replaced by CB-25 which rapidly eluted formic acid from the column. With this modified development procedure, acetic acid recovery was good (100.9 per cent) and formic acid was determined accurately (102.5 per cent) by direct titration.

At the end of an analysis water-Celite 545 chromatograms could be readied for further use by thorough washing with the original developing solvent (benzene). In this way water chromatograms were used two to three times without loss of performance, provided the column surfaces were kept covered with benzene between periods of use.

*Benzene-Aqueous Sulfuric Acid System*.—Failure of separation of *n*-butyric acid from caproic, caprylic, capric, and lauric acids was experienced with the benzene-water chromatogram, since the partition coefficients ( $C_w/C_s$ ) of these acids are too low to permit effective separation. Other solvent systems were therefore investigated by determination of the partition coefficients of *n*-butyric, caproic, caprylic, and lauric acids between their phases.

The partition coefficients were determined by equilibration in 30 ml. glass-stoppered centrifuge tubes. Of the systems investigated, benzene-aqueous sulfuric acid appeared the most promising. Because preliminary results (Fig. 3) showed that the partition coefficients varied with fatty acid concentration, all coefficients were determined on fatty acid solutions which were 0.25 *N* in the benzene phase before equilibration with an equal volume of aqueous sulfuric acid. The coefficients were calculated as concentration in the aqueous phase divided by concentration in the solvent phase.

In Fig. 4 the partition coefficients at room temperature of *n*-butyric, caproic, caprylic, capric, and lauric acids between benzene and sulfuric acid solutions are given. As may be seen in Fig. 4 the concentrations of sulfuric acid between 28 and 36 *N* appeared to hold the most promise for separation of these fatty acids in a benzene-aqueous sulfuric acid partition chromatogram. Low sulfuric acid concentrations, employed in partition chromatograms by Isherwood (5) to suppress ionization of carboxylic acids, have no large effect on partition coefficients.

Benzene-sulfuric acid chromatograms were set up by the procedure previously described, and their effectiveness in separating known mixtures of



fatty acids was determined. From the results obtained and the data presented in Fig. 4 the following was found. (a) Any of the fatty acids studied (*n*-butyric, caproic, caprylic, capric, and lauric) are appreciably delayed in a benzene-sulfuric acid chromatogram if the concentration of sulfuric acid used in the chromatogram is such that the partition coefficient of that fatty acid is at least 10. In order to estimate quantitatively by direct titration a fatty acid after it is removed from the column by further development with benzene, the partition coefficient should be less than 25. If the partition coefficient is greater than 25, the fatty acid will move so

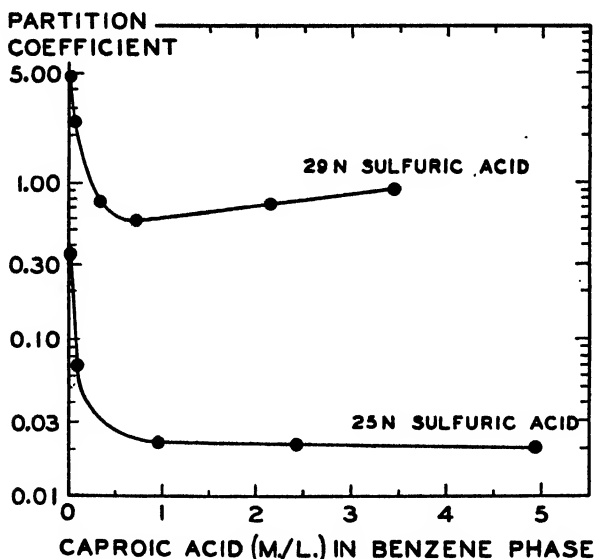


Fig. 3. Partition coefficients of caproic acid between sulfuric acid and benzene at various caproic acid concentrations.

slowly down the column that its zone width at the base of the column will prevent accurate quantitative estimation by direct titration. (b) In order to separate completely any two of the fatty acids studied in a benzene-sulfuric acid chromatogram, the concentration of sulfuric acid used should be such that the partition coefficient of the faster moving fatty acid is less than 5, while the partition coefficient of the slower moving fatty acid is at least 10.

Fig. 5 shows the relation between the position of maximum concentration of any fatty acid in the effluent of a chromatogram and the partition coefficient of the fatty acid between the immiscible phases of the chromatogram. By "position of maximum concentration" is meant the effluent volume at which the fatty acid reaches maximum concentration in the

effluent. As may be seen, this relationship is linear for both macro and micro chromatograms. The intercepts of the lines with the  $Y$  axis are approximately equal to the volumes of mobile phase contained in the respective columns at the time of sample addition. It is obvious that at least this volume of effluent must be collected before any fatty acid can appear in the effluent.

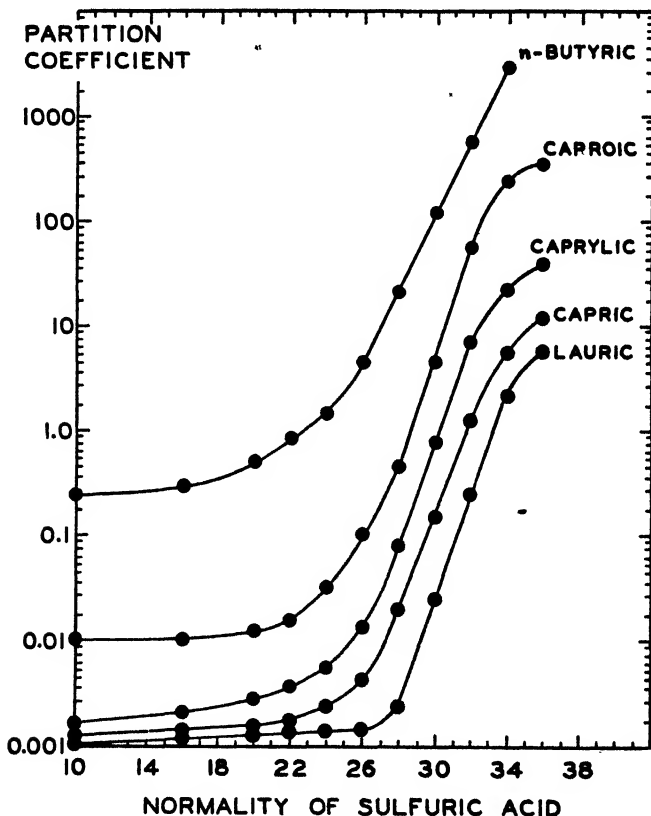


FIG. 4. Partition coefficients of fatty acids between sulfuric acid solutions of varying normality and benzene.

By the use of the data presented in Figs. 4 and 5 it is possible to prepare benzene-sulfuric acid chromatograms capable of quantitative separation of mixtures of any two or three of the following fatty acids: *n*-butyric, caproic, caprylic, and capric acids. Suppose, for example, that a mixture of *n*-butyric, caprylic, and capric acids is to be separated. From Figs. 4 and 5 it may be seen that if a micro chromatogram containing 32.5 *N* sulfuric acid is used *n*-butyric acid, having a partition coefficient of 1000,

will remain on the column, while caprylic acid, having a coefficient of approximately 10, will appear in maximum concentration in the 18th ml. of effluent. Capric acid, having a partition coefficient of about 2.5, will appear at approximately the 7th ml. of effluent. The caprylic and capric acids may be titrated in the effluent, while the *n*-butyric acid may be titrated after removal from the column with water, and steam distillation of the aqueous solution.

It is apparent from the partition coefficients shown in Fig. 6 that with

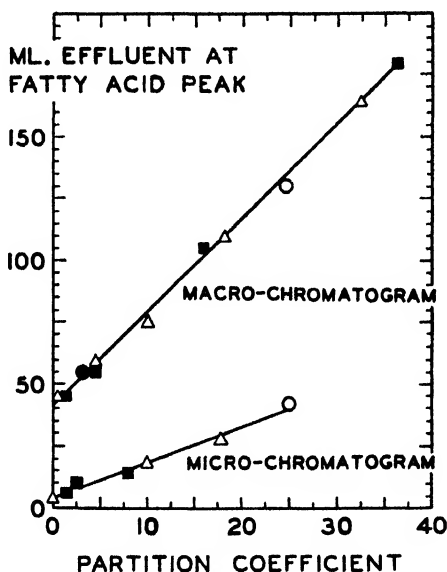


FIG. 5. Position of fatty acid zone in benzene-sulfuric acid partition chromatogram effluent as a function of the partition coefficient of the fatty acid between the phases of the chromatogram. Various fatty acids are designated as follows: *n*-butyric ○, caproic △, caprylic ■, and capric ●.

35 N sulfuric acid good separation of capric and lauric acids is possible on a sulfuric acid chromatogram with 2 per cent Skellysolve B in thiophene-free benzene as the developing solvent. It has been found that such a chromatogram makes possible the separation of capric acid from lauric, myristic, palmitic, oleic, and stearic acids. Attempts to separate these higher acids have not been successful, although it has been found that the use of pure Skellysolve B as a developing solvent for columns containing 32 to 35 N sulfuric acid results in partial separation of adjacent pairs of these acids. Oleic acid passes through such columns without apparent change, since it may be quantitatively titrated in the effluent.

*Estimation of Fatty Acids in Biological Materials*

For the routine estimation of free fatty acids in biological materials, a procedure involving the use of one macro separation column and three micro columns has been developed. This procedure is outlined below.

*Preparation of Sample*—Three preliminary steps are necessary before partition chromatographic methods can be applied to the analysis of saturated fatty acids having as many as 10 carbon atoms in biological materials:

(1) The fatty acids must be completely freed of extraneous material such

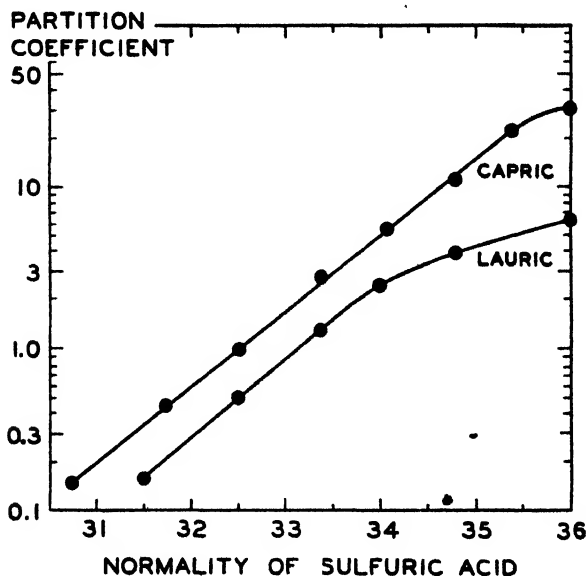


FIG. 6. Partition coefficients of capric and lauric acids between sulfuric acid solutions of varying normality and benzene-2 per cent Skellysolve B.

as proteins and fat. (2) A preliminary quantitative separation of the fatty acids into two groups must be made. (3) The fatty acids in each group must be obtained quantitatively in as small a volume of developing solvent as possible.

To free the fatty acids of extraneous matter, a suitable sample of the biological material to be analyzed is adjusted to pH 2 with 5 per cent sulfuric acid solution, and the free fatty acids liberated are extracted several times with equal volumes of ether. The fatty acids are removed from the ether extracts by several extractions with small volumes of dilute sodium hydroxide. The aqueous solution is concentrated, if necessary, to 100 ml. or less, brought to pH 2 with sulfuric acid, and extracted with three 10 ml.

portions of thiophene-free benzene. The residual aqueous solution, containing only lower fatty acids, is set aside. If necessary, the last traces of aqueous phase are separated from the benzene centrifugally and washed once with 5 ml. of fresh benzene. The benzene solution, 35 ml. or less in volume, is added, without previous saturation with sulfuric acid, to a macro column containing 33 N sulfuric acid. This column is developed with thiophene-free benzene. The separation obtained on this column is shown in Fig. 7. The curve in Fig. 7 was obtained with a fatty acid sample from Cheddar cheese. Similar results are obtained with known fatty acid

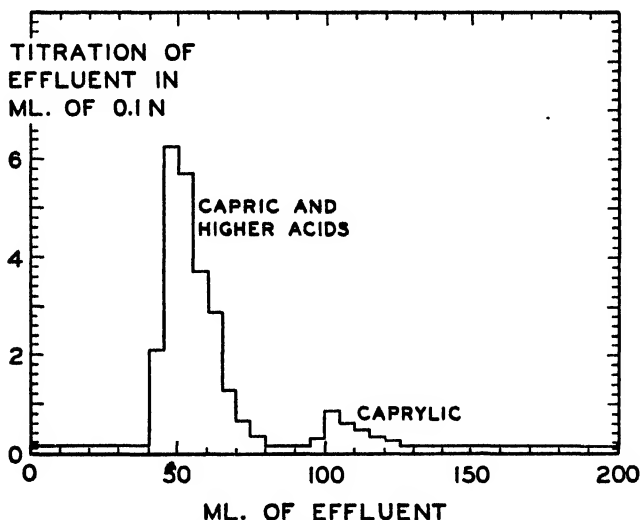


Fig. 7. Separation of higher (capric and higher) and lower fatty acids (caprylic and lower) on a benzene-33 N sulfuric acid partition macro chromatogram. The source of sample was a 26 month-old raw milk Cheddar cheese. In practice, column effluent is collected until the capric and higher fatty acids have passed completely through the column; caprylic and lower fatty acids present are left on the column.

mixtures. In practice, 5 ml. aliquots of column effluent are collected and titrated until the capric and higher acids have passed completely through the column. The caprylic and lower fatty acids present are left on the column, and the combined neutralized effluent fractions containing the higher acids are kept for later analysis.

The contents of the column, including packing, are now removed and combined with the aqueous residues from the benzene extraction. The resulting suspension is brought to pH 8.5 to 9.5 with 10 N sodium hydroxide solution, and the benzene distilled off. After cooling, the residue is brought to pH 2 with sulfuric acid and the volatile acids distilled off by the mag-

nesium sulfate procedure of Friedemann (6). Before distillation the suspension is diluted with water to 275 to 300 ml., and 35 gm. of magnesium sulfate heptahydrate are added. Distillation is continued until magnesium sulfate crystallizes out. The distillate, which contains all of the lower fatty acids (caprylic and below) of the original sample, is titrated to the phenol red end-point with 0.1 N sodium hydroxide. This titration is a measure of the quantity of lower fatty acids present and is used in estimating the amount of sample required in subsequent chromatographic separations and analyses. The solution is concentrated to 10 to 15 ml., mixed with 0.2 to 0.3 gm. of Celite 545, and dried at 110°. After the addition of a few drops of water, the mass is mixed to a uniform damp powder and extracted according to the method of Elsdon (4). Powdered anhydrous sodium acid sulfate (3 to 4 gm.) is added until a fairly dry, pink powder is obtained. This powder is extracted six times with 4 ml. portions of 10 per cent butanol in chloroform (CB-10). The combined extracts are diluted to 25 ml. and kept for later analysis.

The neutralized benzene effluent, containing the higher fatty acids, is evaporated to dryness and extracted with CB-10 in the same manner as for the lower fatty acid fraction.

*Chromatography of CB-10 Extracts*—An aliquot (2 ml. or less) of the extract containing the lower fatty acids is analyzed on a micro chromatogram with water as the non-mobile phase. Thiophene-free benzene, CB-10, and CB-25 are used in turn as developing solvents. As may be seen from Fig. 2, formic, acetic, and propionic acids appear separately, while butyric and higher acids appear as a single zone. Another aliquot of the same CB-10 extract is analyzed on a micro chromatogram in which the non-mobile phase is 30.5 N sulfuric acid and the developing solvent is thiophene-free benzene. In this column, caproic and caprylic acids appear as separate zones, while butyric and lower fatty acids remain on the column. Thus, formic, acetic, propionic, caproic, and caprylic acids are directly determined. Butyric acid may be determined by difference or may be resolved as a separate zone with a benzene-27.5 N sulfuric acid micro chromatogram. Fatty acids containing an odd number of carbon atoms (aside from formic and propionic acids) have not been encountered.

The CB-10 extract containing capric and higher fatty acids is used for the direct determination of capric acid with a micro chromatogram containing 35 N sulfuric acid. Thiophene-free benzene containing 2 per cent Skellysolve B is used as the developing solvent. On this column capric acid appears as a separate zone, while fatty acids higher than capric appear as a single zone and can be determined only as a group.

TABLE I

*Recovery of Individual Fatty Acids in Known Mixtures by Partition Chromatography*

Fatty acid	Mixture I		Mixture II		Mixture III		Mixture IV	
	Fatty acid present	Fatty acid recovered	Fatty acid present	Fatty acid recovered	Fatty acid present	Fatty acid recovered	Fatty acid present	Fatty acid recovered
	microequiv- alents	microequiv- alents	microequiv- alents	microequiv- alents	microequiv- alents	microequiv- alents	microequiv- alents	microequiv- alents
Formic . . . .	80	83.1	20	21.6	20	20.9	110	115
Acetic . . . .	80	80.6	20	20.2	20	20.9	110	112
Propionic . .	80	78.2	200	197	20	19.0	20	19.6
<i>n</i> -Butyric . .	80	79.7	20	21.4	20	19.9	110	110.5
Caproic . . .	80	77.8	200	195	20	18.1	20	19.1
Caprylic . . .	80	79.4	20	19.5	20	19.6	110	108
Capric . . . .	80	77.5	200	190	20	18.4	20	20.4
Lauric . . . .	80	76.8	20	37.4	50	237	200	189
Myristic . . .					50			
Palmitic . . .					50			
Oleic . . . . .					50			
Stearic . . . .					50			

TABLE II

*Recoveries of Fatty Acids Added to Butter Fat*

Fatty acid	Butter Fat I*				Butter Fat II†			
	Fatty acid found	Fatty acid recoveries			Fatty acid found	Fatty acid recoveries		
		Fatty acid added	Total fatty acid found	Recovery of added fatty acid		Fatty acid added	Total fatty acid found	Recovery of added fatty acid
	microequiv- alents	microequiv- alents	microequiv- alents	per cent	microequiv- alents	microequiv- alents	microequiv- alents	per cent
Formic	0	750	778	103.9	0	150	154	102.8
Acetic	0	750	744	99.1	0	150	148	98.7
Propionic	0	750	726	96.9	0	150	146	97.4
<i>n</i> -Butyric	397	750	1178	104.2	381	150	532	100.7
Caproic	146	750	852	94.2	153	150	297	96.0
Caprylic	83.0	750	822	98.5	77.1	150	226	99.4
Capric	130	2000	2026	94.7	128	2000	2070	97.1
C <sub>12</sub> and higher	3268	1000 (Lauric) 1000 (Oleic)	5104	91.7	3914			

\* Individual fatty acids to be recovered were added to separate samples of butter fat.

† All fatty acids to be recovered were added to the same butter fat sample.

### *Reliability of Method*

*Recoveries on Known Mixtures*—Known aqueous mixtures of the sodium salts of fatty acids have been subjected to the entire preparatory and

analytical procedure described above. The recoveries of individual fatty acids of four such mixtures are given in Table I.

*Butter Fat Analyses and Recoveries of Added Fatty Acids*—In Table II fatty acid analyses and added fatty acid recoveries for two butter fat samples are presented. While no component acid analyses of these two butter fats by recognized methods are available for comparison, it may be noted that the results for individual fatty acids obtained by partition chromatography fall well within the normal limits of fatty acid analyses on butter fat (7). As may also be seen, recoveries of added fatty acids are adequate for most purposes.

#### SUMMARY

1. Sulfuric acid (27 to 35 N) is a better solvent for fatty acids of intermediate chain length than water, and hence may be used as the non-mobile phase in partition chromatograms for the analysis of fatty acid mixtures. By the use of both water and sulfuric acid partition chromatograms, quantitative separation of formic, acetic, propionic, *n*-butyric, caproic, caprylic, and capric acids is possible. Higher fatty acids do not interfere.

2. The partition coefficient data presented facilitate the design of partition chromatograms for separation of any mixture of the above acids.

3. Detailed procedure is given for the quantitative analysis of fatty acids in biological materials. For accurate analysis of any one fatty acid, more than 10 microequivalents of that acid must be present on the column.

4. Fatty acids in known mixtures, or fatty acids added to butter fat samples, were recovered with a maximum error of 8 per cent.

*Addendum*—After this paper had been submitted, a publication appeared (8) describing a methanol-isooctane partition chromatogram for the separation and determination of saturated fatty acids of intermediate chain length. Preliminary work has indicated that such chromatograms might be used in conjunction with the procedures described in the present paper.

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# THE DETERMINATION OF IRON IN SMALL VOLUMES OF BLOOD SERUM\*

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In an effort to find practical means of distinguishing iron deficiency anemias from other types in large population groups, an investigation was made of the possibility of measuring iron in an amount of blood serum obtainable from the finger. Iron deficiency anemias are characterized by low serum iron, while other states of diminished hemoglobin formation show normal or even high values for serum iron (1-4). The prevalence of iron deficiency anemias in large population groups has not been widely investigated, owing partly to the difficulty of obtaining necessary quantities of blood for analysis and partly to the troublesomeness of existing methods. Present methods require 1 to 10 ml. of blood serum and time-consuming techniques.

Most widely used of the methods for iron in blood serum are those involving color reactions of ferrous iron with  $\alpha, \alpha'$ -bipyridyl (5, 6) or *o*-phenanthroline (4, 7) and of ferric iron with thiocyanate ion (1, 8-10). Of these three reagents, thiocyanate ion appeared to offer advantages for a simple microprocedure. Both bipyridyl and *o*-phenanthroline require several steps for their use (pH adjustment and reduction of ferric to ferrous iron prior to color formation) which it is possible to avoid by using a thiocyanate procedure. It has been found possible, with thiocyanate ion, to measure iron with satisfactory precision in 20 c.mm. of serum. In this method, which is described below, acid and thiocyanate ion are added as one reagent to the serum and the iron thiocyanate is extracted directly from the mixture with isoamyl alcohol. This avoids the preparation of serum filtrates and improves the extraction of iron from the serum proteins.

In addition to a description of the procedure, illustrative data are presented; *viz.*, the daily and hourly variation in serum iron in several normal persons.

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## EXPERIMENTAL

*Reagents*—All water used is carefully redistilled from glass and stored in Pyrex glass-stoppered bottles.

1.  $\text{NH}_4\text{SCN}$ , 27 per cent. Traces of iron are removed by adding 0.05 ml. of concentrated  $\text{HCl}$  per 100 ml. and extracting with a 3:1 mixture of isoamyl alcohol and ethyl ether until no pink color is visible. Stored at  $4^\circ$  this reagent is satisfactory for several months.

2. Hydrochloric acid, 1.35  $N$ , prepared from redistilled 6  $N$  (constant boiling) acid.

3. Isoamyl alcohol, redistilled, saturated with water, and stored at  $4^\circ$ . On the day used, to 10 ml. is added 0.1 ml. of 0.2 per cent  $\text{H}_2\text{O}_2$  prepared from 30 per cent  $\text{H}_2\text{O}_2$  (superoxol).

4. Complete reagent. Equal volumes of 27 per cent  $\text{NH}_4\text{SCN}$  and 1.35  $N$   $\text{HCl}$  are mixed for each series of determinations. This reagent keeps not more than 2 hours.

5. Standard iron solution. A stock, 100 mg. per cent, iron solution is prepared from iron wire (Bureau of Standards) by means of redistilled  $\text{HCl}$  and a few drops of redistilled nitric acid. A working standard of 200  $\gamma$  per cent is prepared in 0.01  $N$   $\text{HCl}$ .

6. Ascorbic acid, 30 per cent in ethylene glycol. Warming to  $60^\circ$  is necessary to dissolve completely; higher temperatures result in discoloration. Preparation of fresh solution daily is recommended.

*Apparatus for 20 C.mm. of Serum—*

1. Constriction pipettes 2, 20, 60, and 90 c.mm. (11, 12) and two fine tipped constriction pipettes of about 80 c.mm. capacity for transferring samples in and out of the spectrophotometer cuvettes.

2. Blood-collecting tubes. Melting point capillaries, 1.5 to 2.0 mm. outside diameter, 7 to 10 cm. long, *e.g.*, No. 34500, Kimble Glass Company, Vineland, New Jersey. These and the serological tubes listed below are cleaned by boiling in half concentrated nitric acid, rinsing in distilled water, boiling in distilled water, rinsing finally with redistilled water, and dried in an oven at  $45$ – $50^\circ$ .

3. Serological tubes 6  $\times$  50 mm.; *e.g.*, Kimble, No. 45060.

4. Wooden or plastic racks for tubes.

5. Glass-stoppered flasks for reagents. These are cleaned in acid and rinsed thoroughly with distilled and glass-redistilled water.

6. Centrifuge fitted with cups suitable for small tubes.

7. Beckman spectrophotometer adapted to small volumes (13). Micro cells of 2 to 2.4 mm. inner width are appropriate.

8. High speed hand drill with a flattened nail for violently agitating the samples to effect the extraction of the aqueous phase with isoamyl alcohol (14).

*Apparatus for 0.150 Ml. of Serum—*

1. Constriction pipettes, 0.015, 0.150, 0.45, and 0.6 ml., and two transfer pipettes of about 0.5 ml. capacity.
2. Blood-collecting tubes. Pyrex tubing 4 mm. outside diameter, 10 cm. long; cleaned as described above.
3. Sleeve stoppers (vaccine caps) for stoppering the blood-collecting tubes. The outside sleeve is cut off, and the inner core is used. No. 1A-66F, West Company, Phoenixville, Pennsylvania.
4. Pyrex test-tubes, 5 ml. "
5. Other equipment as described above. A Coleman junior (model 6-A) spectrophotometer with a special adapter may be used for this volume (12).

*Procedure*

*Collection of Samples*—Special care must be taken to have the surface of the finger clean. It is then wiped with 80 per cent alcohol and incised with a new, clean, Bard-Parker scalpel blade (No. 11). The first drop of blood is discarded by wiping with a *clean towel*. The small samples, *e.g.*, for 20 c.mm. of serum, are collected and sealed in the small capillary as previously described (15). Volumes up to 150 c.mm. of serum (300 c.mm. of blood) are more conveniently collected by the use of the larger tubes. By squeezing the finger this amount of blood is easily obtainable. If necessary, the hand should be warmed. The first few mm. of the dry end of the tube are coated with vacuum wax or vaseline and a vaccine cap firmly pressed into place after filling. The unwaxed end of the tube is closed by another cap. Before centrifuging, the cap on the waxed end of the tube is taped on with adhesive tape. After sealing, the samples are allowed to clot, are centrifuged, and the serum is collected at once and stored in iron-free tubes, preferably frozen, until analyzed.

*Analysis*—It is, of course, necessary to work in rooms as free as possible from dust and to take every precaution to avoid contamination of samples and clean vessels by covering them whenever possible with dust-proof materials. To 20 c.mm. of serum in a 6 × 50 mm. tube are added 60 c.mm. of the "complete reagent." The sample is well shaken at once by tapping to obtain a finely divided precipitate from which iron can be extracted. 90 c.mm. of isoamyl alcohol containing  $H_2O_2$  are added. The tube is covered with Parafilm, and mixed violently by vibration with the high speed motor and nail ("Apparatus," item (8)).

Blanks and standards are prepared at the same time by substituting either water or standard iron solution for the serum.

The tubes are chilled in centrifuge cups to 4–10° and then centrifuged at 3000 R.P.M. for 10 minutes. Approximately 80 c.mm. of the upper

isoamyl alcohol layer are pipetted carefully into the Beckman cell with care to avoid contamination from the precipitate interface or from the water layer. (To clean the narrow Beckman cells, allow them to stand for 10 or 15 minutes filled with 1 N HCl, rinse well with redistilled water, and dry with alcohol and ether. Finally, one of the reagent blanks is used for rinsing the cells.) With isoamyl alcohol in the reference cell, the optical density of the sample is read at 520  $m\mu$  ( $R_1$ ). The entire sample is removed from the cell with a fine pipette and delivered into a clean tube. 2 c.mm. of the 30 per cent ascorbic acid reagent are added and the solution is mixed. After 10 to 60 minutes, the optical density of the solution is again measured ( $R_2$ ).

In practice, a series of samples is read, removed from the cells, and reduced with ascorbic acid. The tubes are covered with Parafilm and allowed to stand until the completion of the original readings on a series. Then the optical density of the reduced solutions is determined.

*Calculation*—Since the same pipettes are used for serum and for iron standard solutions, the volume of the pipettes does not enter the calculations.

$$R_1 - R_2 = R_x$$

$$\frac{(R_x - R_{\text{blank}}) \times 200}{R_{\text{standard}} - R_{\text{blank}}} \times \frac{100}{90} = \gamma \% \text{ Fe}$$

The factor 100/90 is introduced to correct for the incomplete recovery (90 per cent) of iron from serum.

#### DISCUSSION

*Extraction of Iron from Serum*—For the conversion of iron into a determinable form, two types of methods have been employed previously: (1) ashing and (2) preparation of protein-free filtrates. The various ashing methods give total iron values for serum without correction for the possible contribution of iron due to hemolysis. Since hemolysis is likely to occur to a slight degree even under very favorable conditions, ashing methods for large numbers of samples were considered unsatisfactory.

Methods depending on precipitation of the protein and removal of iron in the filtrate were found to be unsatisfactory on the micro scale. The recovery of added iron was low and irregular. It was found possible to obtain better recovery of iron and with fewer steps by adding acid and  $\text{NH}_4\text{SCN}$  as one reagent to the serum and extracting the  $\text{Fe}(\text{SCN})_3$  directly with isoamyl alcohol. Since irregularities in results were found when trichloroacetic acid was used to precipitate the proteins, it was omitted from the reaction mixture. The thiocyanic acid itself causes a considerable precipitation of the proteins.

The concentrations of HCl and  $\text{NH}_4\text{SCN}$  and peroxide were chosen to give maximal extraction of iron and color development and minimal blank values. Increasing any of these three reagents beyond the given concentration tends to cause an increase in the blank.

*Correction for Acid Hematin*—In addition to  $\text{Fe}(\text{SCN})_3$ , acid hematin is

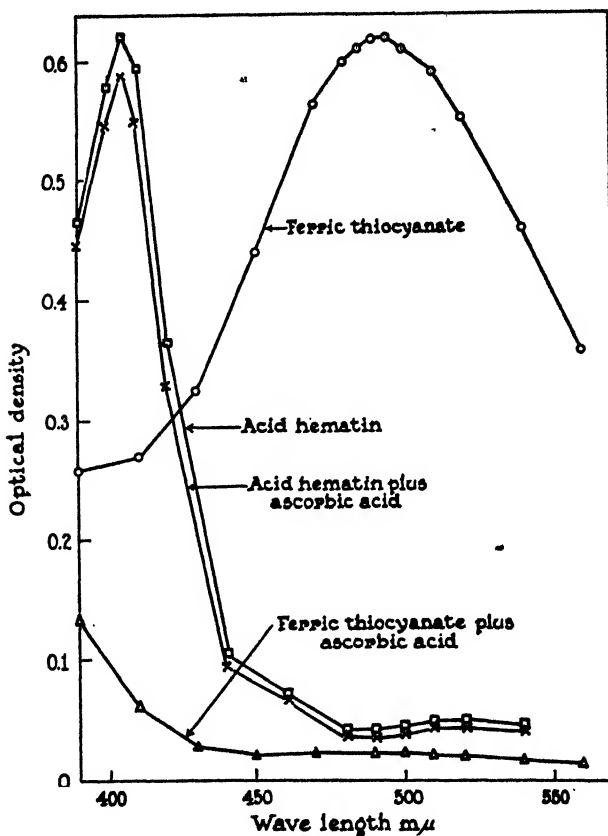


FIG. 1. Absorption curves of isoamyl alcohol extracts of aqueous solutions: acid hematin, acid hematin plus ascorbic acid, ferric thiocyanate, ferric thiocyanate plus ascorbic acid.

unfortunately also extracted into isoamyl alcohol together with other unknown materials present in serum which absorb light at the wave-length used for measurement ( $520\text{ m}\mu$ ). A means was, therefore, sought to eliminate, correct for, or reduce to a minimum this interference.

Since acid hematin has an absorption 12 times greater at  $400\text{ m}\mu$  than at  $520\text{ m}\mu$  (Fig. 1), it is possible to achieve an approximate correction for acid

hematin by measuring the absorption of the isoamyl alcohol extracts at 400  $m\mu$  and calculating the contribution of hematin to the absorption at 520  $m\mu$ . However, other materials extracted from serum make the proper correction factor somewhat uncertain and, therefore, this procedure unsatisfactory.

The selective destruction of the color of  $\text{Fe}(\text{SCN})_3$  without changing the absorption of the interfering materials was another possibility. The addition of ammonia decolorized  $\text{Fe}(\text{SCN})_3$  but also caused a change in the color of acid hematin. Reagents such as stannous chloride and sodium arsenite reduced the  $\text{Fe}(\text{SCN})_3$  but proved unsatisfactory for other reasons. However, it was found that minute quantities of ascorbic acid rapidly reduced  $\text{Fe}(\text{SCN})_3$  in isoamyl alcohol solution and that acid hematin was not affected, particularly in the 500  $m\mu$  region (Fig. 1). A 30 per cent solution of ascorbic acid in ethylene glycol proved to be a satisfactory reducing agent because of the low volatility of the solvent and the relatively small volume of solution required for the reaction.

In addition to acid hematin there may be a slight contribution to the blank from other substances present in serum. These substances show minimal absorption at 520  $m\mu$  (Fig. 2). Although the absorption of  $\text{Fe}(\text{SCN})_3$  is somewhat less than its maximum at this wave-length, absorption at 520  $m\mu$  was chosen as best for the serum iron measurements in order to keep the blank values as low as possible.

The difference curves (Fig. 2) furnish further evidence that only  $\text{Fe}(\text{SCN})_3$  is changed by reduction with ascorbic acid. The absorption curves for  $\text{Fe}(\text{SCN})_3$  extracted from serum and for the same extract after addition of ascorbic acid were measured between 420 and 560  $m\mu$ . The difference curve represents the change in absorption on reduction. This difference curve is compared with a difference curve calculated from the absorption curve of  $\text{Fe}(\text{SCN})_3$ . It can be seen that the actual and calculated difference curves nearly coincide.

*Standards and Recoveries*—Proportionality of the  $\text{Fe}(\text{SCN})_3$  color produced at low concentrations of iron in the micromethod is satisfactory (Fig. 3). Iron standards containing 95, 184, and 328  $\gamma$  per cent of Fe were used in 20 c.mm. quantities (19, 37, and 66  $m\gamma$  of Fe per sample) for these measurements.

Similar concentrations of iron standards were added to serum for recovery experiments (Table I). Whether or not the reagent stands in contact with the serum before extraction with isoamyl alcohol seems to make no significant difference in the recovery, which averaged 89 per cent (Table I). Additional tests have shown that recovery was not increased by allowing the serum to stand with one-fourth its volume of 6 N HCl for 15 minutes prior to the addition of  $\text{NH}_4\text{SCN}$  and isoamyl alcohol. The peroxide is added to convert and maintain iron in the ferric (III) state.

**Reproducibility**—Separate samples of blood from each of four fingers of the same hand taken in consecutive order were analyzed in triplicate (Table II). The standard deviation among the twelve analyses was 3.6  $\gamma$  per cent, which agrees with other replicate analyses. The agreement between the averages for each finger is about that expected from the standard deviation, which suggests that the method of obtaining blood from the finger need not introduce additional errors. This is borne out by comparison of analysis on blood serum from the finger and from the vein

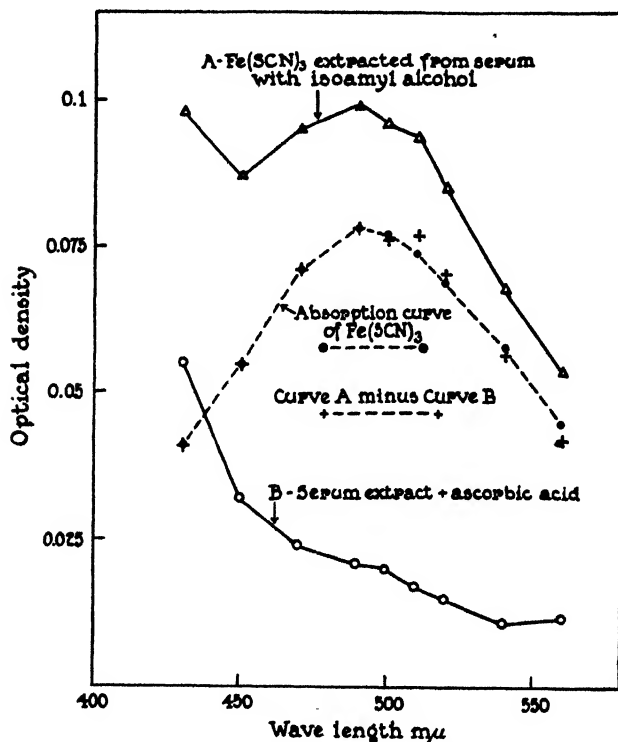


FIG. 2. Absorption curves (Curve A) of  $\text{Fe}(\text{SCN})_3$  extracted from serum with isoamyl alcohol and (Curve B) of this extract reduced with ascorbic acid and difference curves obtained by subtracting the values for Curve B from Curve A and by calculating the difference curve from the absorption curve of  $\text{Fe}(\text{SCN})_3$ .

(Table III). It is indicated in Table III that simple cleansing of the finger with alcohol is more effective in preventing contamination of the sample than attempting to coat the finger with vaseline, etc.

**Daily and Hourly Fluctuations**—Fig. 4 records the hourly variations observed in the serum levels of three subjects during three morning periods. In each instance the subjects had been without food for about 15 hours



prior to the test to avoid possible alimentary effects. Rather marked fluctuations were observed without any definite trend. The greatest

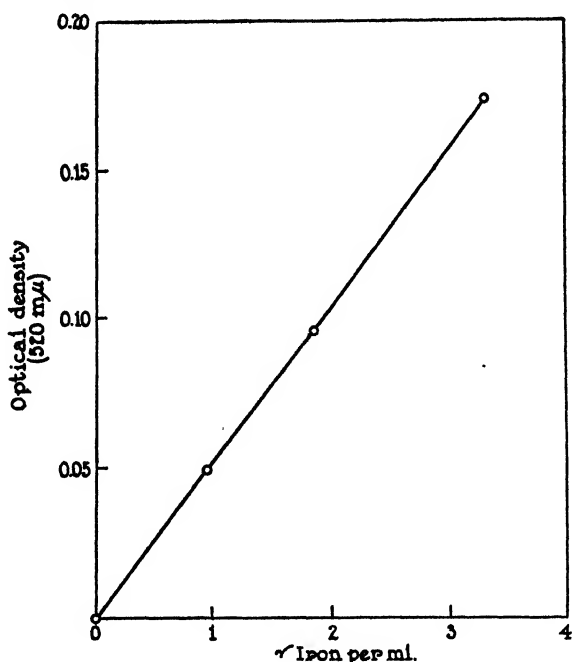


FIG. 3. Curve showing proportionality between the concentration of iron in standard solutions and the optical density of the reaction product ( $\text{Fe}(\text{SCN})_3$ ) as measured in the isoamyl alcohol layer.

TABLE I  
*Recovery of Iron Added to Serum*

Treatment	Serum Fe, initial	Added Fe	Total Fe, found	Recovery
	$\gamma$ per 100 ml. serum	$\gamma$ per 100 ml. serum	$\gamma$ per 100 ml. serum	per cent
Standing with reagent 15 min.	54	100	141	87
before addition of isoamyl alcohol	53	202	234	90
	50	396	400	89
Reagent followed immediately	48	100	134	86
by isoamyl alcohol extraction	48	202	234	91
	48	396	400	89

change in any one 3 hour period was 40  $\gamma$  per cent, the smallest was 6, and the average was 20  $\gamma$  per cent. In no case, however, did any sample fall below 65 or rise above 122  $\gamma$  per cent. Heilmeyer and Plötner (4)

have reported a decrease in serum iron after 6 hours fasting, with a subsequent rise of 25 per cent after 24 hours. Changes in the serum iron levels also occur on different days, as noted by Skouge (2) and more recently by Høyer (16). The latter describes a fall in serum iron from morning to evening of an average of 42  $\gamma$  per cent, and daily and weekly average varia-

TABLE II  
*Serum Fe in Consecutive Samples of Finger Blood*

	Finger 1	Finger 2	Finger 3	Finger 4
Fe, $\gamma$ %	94 98 96	94 98 89	92 94 96	102 100 94
Average .....	96	94	94	99
" 12 analyses .....				96
Standard deviation .....				3.6

TABLE III  
*Comparison of Iron in Blood Serum from Vein and Finger*

Source of serum	Fe $\gamma$ per 100 ml.	Source of Serum	Fe $\gamma$ per 100 ml.
Vein	99 107 112	Finger coated with mineral oil	185 139 141
Average .....	106		138
Finger cleaned with alcohol	113 113 120	Finger coated with vaseline	128 127
Average	115		128

tions of 30  $\gamma$  per cent. Vahlquist (3) obtained 36  $\gamma$  per cent lower serum iron at 6.00 p.m. than at 8.00 a.m. on fifteen male subjects.

Periodically during a period of 1 month, at 11.00 a.m., blood serum was obtained from each of four adult women and analyzed for iron by the proposed micromethod. The individual averages were 92, 94, 100, and 109  $\gamma$  per cent. The standard deviations were, respectively, 24, 23, 23, and 21  $\gamma$  per cent (Table IV). The lowest value observed was 60 and the highest 155  $\gamma$  per cent. No correlation of the iron values with the menstrual cycle

## DETERMINATION OF IRON IN BLOOD SERUM

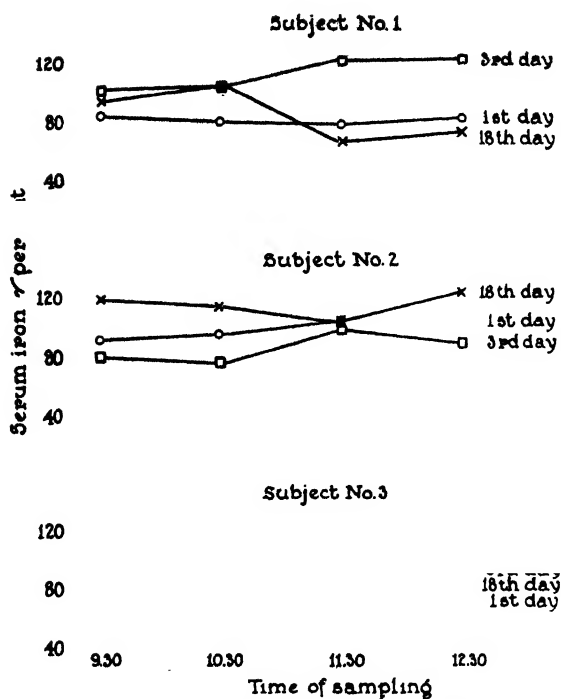


FIG. 4. Hourly serum iron levels in fasting subjects

TABLE IV  
*Serum Fe Determinations on Four Subjects During 1 Month*

Time interval	Subject A	Subject B	Subject C	Subject D
<i>days</i>	<i>γ per 100 ml.</i>	<i>γ per 100 ml.</i>	<i>γ per 100 ml.</i>	<i>γ per 100 ml.</i>
1	126	90	110	78
5	64	120	155	111
7	75	94	140	138
10	81	127	91	117
12	96	107	98	130
14	135		96	100
17	83	73	64	127
19	118	72	76	111
21	90	60	110	123
24	73	111	81	80
26	77	88	100	128
28	73	93	85	73
31	101			118
Average.....	92	94	100	110
S.D.....	±24	±23	±23	±21

was observed. These averages and fluctuations concur with the data in the literature obtained with macro serum iron methods.

It is apparent from these measurements and those in the literature that the serum iron levels may undergo considerable hourly and daily variation. This must surely be taken into account in interpreting data designed to reveal nutritional status with respect to iron. On the basis of knowledge now available on the subject, the following conclusions seem reasonable. Serum iron values less than 60  $\gamma$  per cent are rarely found in well nourished persons and, therefore, in the absence of infection or other pathology (17) such low values can probably be interpreted as indicating an iron deficiency. Conversely, values above 60  $\gamma$  per cent indicate an adequate iron supply. Since factors other than iron supply lead to rather wide fluctuations in serum values, such analyses cannot be safely used to estimate iron intake except on a rather rough scale. This limitation notwithstanding, serum iron analysis, if a suitable method were available, would seem to provide a useful and practical means of determining the occurrence of iron deficiency in large population groups. It is felt possible that the proposed micromethod may serve this function.

#### SUMMARY

1. A simple method is described for the determination of iron in 20 c.mm. of blood serum with ferric thiocyanate as the colored compound measured. Iron added to serum is consistently recovered to the extent of about 90 per cent. The standard deviation of replicates is 3 to 5  $\gamma$  per cent. Since blood serum from the finger can be used and since one analyst can perform twenty-five to fifty analyses in a day, the method appears suitable for nutritional surveys.

2. Hourly variations in serum iron values averaging 20  $\gamma$  per cent were observed during 3 hour periods for three well nourished persons. The variations over a period of a month for four adult women were greater but seemed to be restricted within the limits of 60 to 155  $\gamma$  per cent. Even with this variation it is felt that, since serum iron of less than 60  $\gamma$  per cent is rare among well nourished persons, serum iron analysis by the method described provides a useful and reliable means for determining the occurrence of iron deficiency in large population groups.

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# THE EFFECT OF PYRIDOXINE DEFICIENCY ON THE TRYPTOPHAN-NIACIN TRANSFORMATION IN RATS\*

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Investigations conducted by Lepkovsky and his coworkers (1), Cartwright *et al.* (2), Schweigert and Pearson (3), and Rosen, Huff, and Perlzweig (4) indicate that pyridoxine plays a rôle in the conversion of tryptophan to niacin derivatives in rats, mice, and swine. Rosen, Huff, and Perlzweig observed that in pyridoxine-deficient rats the urinary excretion of N<sup>1</sup>-methylnicotinamide after ingestion of tryptophan did not return to normal levels even after 2 weeks of refeeding with pyridoxine. They suspected that certain permanent changes might have occurred to the pyridoxine-deficient rats which impaired the conversion mechanism. The question still remains whether the transformation occurs in the intestinal tract by bacterial synthesis or metabolic processes in the animal body. Kohn (5, 6), Handler (7), and their collaborators have shown that when niacin was ingested by human subjects, or added to the blood *in vitro*, it was quickly synthesized into pyridine nucleotides (coenzymes I and II) by the red blood cells. Therefore, it was thought of interest to investigate the effect of intravenous injection of tryptophan on the fluctuation of total pyridine nucleotides in the erythrocytes in normal and pyridoxine-deficient rats.

## EXPERIMENTAL

Three lots of albino rats were used in this study. These were eighteen young adult males weighing between 203 and 258 gm. at the beginning of the experiments, twelve females weighing between 261 and 283 gm., and ten young males between 72 and 98 gm. in weight. One-half of the adult animals of each sex was given the pyridoxine-deficient diet and the other half the control diet. Of the young animals, seven were made deficient and three served as controls. All of these animals were kept in individual cages with food and water supplied *ad libitum*.

Pyridoxine deficiency was induced by omitting pyridoxine from the diet and including the antivitamin desoxyypyridoxine (8, 9). The composition

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of the pyridoxine-deficient diet is given in Table I. The control diet was the same except that 500  $\gamma$  of pyridoxine hydrochloride were included per 100 gm. of diet.

The young male rats began to show signs of acrodynia after being on the pyridoxine-deficient diet for about 5 weeks. The paws became reddened, squamous, and swollen, and the region about the nose was red and eroded with inflammatory secretion. Several of the young rats developed a scaly tail. Loss of appetite and emaciation were generally noted among these deficient animals. At 7 weeks, some of the adult males on the deficient diet also developed typical dermatitis. Most of the deficient animals exhibited nervousness and fright, and one of them died of motor paralysis

TABLE I  
*Composition of Pyridoxine-Deficient Diet*

Ingredients	Per 100 gm.	Ingredients	Per 100 gm.
	gm.		mg.
Casein, vitamin-free, Labco	18.0	<i>p</i> -Aminobenzoic acid	10
Salt Mixture IV*	4.0		$\gamma$
Sucrose	72.7	Thiamine hydrochloride	400
Corn oil	5.0	Riboflavin	800
L-Cystine	0.3	Ca pantothenate	2500
Choline chloride	0.1	Folic acid	100
	mg.	Biotin	20
$\alpha$ -Tocopherol	15	Desoxypyridoxine	2000
Inositol	20		drops
		Oleum percomorphum	15

\* Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **138**, 459 (1941).

preceded by ataxia. Several others succumbed to pneumonia and septic infections. The main feature on autopsy of pyridoxine-deficient rats was marasmus and generalized atrophy of the lymphoid tissues. In order to keep these rats alive and maintain the deficient state for study, desoxypyridoxine was withdrawn from their rations and its casein content increased to 25 per cent after the 7th week of the experiment.

Determination of total pyridine nucleotides in the erythrocytes was made by the alkali-acetone condensation method of Levitas *et al.* (10), somewhat modified for the use of a small quantity of blood specimen. 0.4 ml. of the whole blood was laked drop by drop in a small test-tube containing 0.8 ml. of a 25 per cent trichloroacetic acid solution and 2.8 ml. of distilled water. After standing for about 2 minutes, the mixture was centrifuged and filtered, and 0.3 ml. of the filtrate was used for each tube in the analysis according

to the original method described by the above authors. Readings were made in a Lumetron fluorometer with the quinine sulfate standard (0.3  $\gamma$  per ml.); the galvanometer was set at 100. Hematocrits were determined simultaneously, mostly in duplicate, with Van Allen micro hematocrit tubes.

Separate determinations of fluorescent material in the plasma and in the erythrocytes were made on several samples taken from two animals before and after the injection of tryptophan. It was found that the plasma contains only one-tenth to one-fifteenth, with an average of 9 per cent, of the total fluorescent material in the whole blood. Hence, when whole blood was analyzed, the fluorescent material in the erythrocytes was computed by subtracting 9 per cent from the value obtained with the whole blood. This was found to be in agreement with the values determined directly from washed erythrocytes in the subsequent experiment. The fluorescent material per ml. of erythrocytes was calculated from the hematocrit obtained at each period. The recovery values of 0.5  $\gamma$  of N<sup>1</sup>-methylnicotinamide added to the blood filtrate were in most instances 38 divisions per microgram on the Lumetron fluorometer, which was similar to those reported by Levitas *et al.* Therefore, the diphosphopyridine nucleotide-N<sup>1</sup>-methylnicotinamide proportionality factor,  $15/38 = 0.4$ , seems to be applicable and was used in the computation of total pyridine nucleotides in the erythrocytes and expressed as diphosphopyridine nucleotide (DPN).

In the experiment on the pyridine nucleotide synthesis by erythrocytes following the injection of niacin, the pyridine nucleotide content in the red blood cells was determined from erythrocytes washed several times with Ringer's solution and resuspended in the Ringer's solution to 1 ml. volume. This suspension was laked in trichloroacetic acid solution and analyzed in the manner described above, and the pyridine nucleotide per ml. of erythrocytes calculated from the hematocrit.

N<sup>1</sup>-Methylnicotinamide in the urine was determined by the fluorometric method of Huff and Perlzweig (11). Xanthurenic acid excretion in the urine was measured by the photometric method described by Rosen, Huff, and Perlzweig (4) based on the green ferric salt formation in the alkaline urine suggested by Lepkovsky *et al.* (1).

*Fluctuation of Total Pyridine Nucleotides in Erythrocytes after Intravenous Injection of Tryptophan in Normal and Pyridoxine-Deficient Rats*—1 ml. of a freshly prepared sterile solution of L-tryptophan containing 50 mg. per ml., in 0.12 N NaOH, was injected into the tail vein of the rat. About 0.5 to 0.7 ml. of blood specimen was taken by heart puncture just before and every 2 hours after the injection of tryptophan, and total pyridine nucleotide and hematocrit determined. The results of the nucleotide determinations are summarized in Fig. 1. In the normal group, some of the



animals died of heart puncture due to pericardial hemorrhage before complete data were obtained. It is interesting that the mortality of heart puncture from pericardial hemorrhage was higher among the normal than the pyridoxine-deficient rats.

In the normal rats, after intravenous injection of tryptophan, the pyridine nucleotide level began to rise in 4 hours, reached its peak between 8 and 10 hours, and decreased gradually thereafter to near its initial level after 24 hours. The results of separate analysis of plasma and red blood cells indicated that the plasma portion of the fluorescent material did not increase

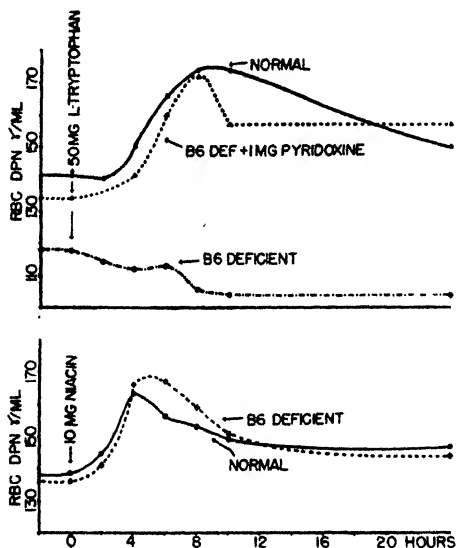


FIG. 1. The effect of tryptophan and niacin injections upon the erythrocyte DPN level in normal and pyridoxine-deficient rats. Each point represents the average of the values obtained from five rats in the upper three curves and from three rats in the lower two curves.

after the injection of tryptophan, but rather tended to be slightly lower than the initial levels as more blood was withdrawn. Apparently, any increase in the fluorescent material in the blood after injection of tryptophan was in the erythrocytes and not in the plasma.

The pyridoxine-deficient animals had a low average initial level of pyridine nucleotides in the erythrocytes, and in contrast to the rise in nucleotides observed in normal animals after the tryptophan injection, there was a slight decline, probably due to the loss of blood.

The average initial hematocrit was 47.6 for the normal, and 46.6 for the pyridoxine-deficient rats. The hematocrit decreased at an average rate of approximately 7 per cent its total value after each heart puncture, and

there was no significant difference between the normal and the pyridoxine-deficient groups of animals.

*Response of Pyridoxine-Deficient Rats to Injection of Pyridoxine on Restoration of Their Ability to Convert Tryptophan into Nicotinic Acid Derivatives*—The effect of subcutaneous injection of 1 mg. of pyridoxine hydrochloride 1 hour prior to the intravenous injection of 50 mg. of L-tryptophan in the pyridoxine-deficient rats was examined. After the injection of pyridoxine, the animals immediately increased their appetite and began to feed prodigiously. Blood specimens taken just before the injection of tryptophan gave an average initial value of pyridine nucleotides in the erythrocytes between those of the normal and the deficient rats without previous injection of the vitamin. The highest average level was reached 8 hours after tryptophan was administered and maintained at a relatively high level even after 24 hours. Apparently, with the increased appetite, sufficient tryptophan was obtained from the casein (25 per cent) in the diet to keep the pyridine nucleotides at this high level.

*Synthesis of Pyridine Nucleotides by Erythrocytes Following Subcutaneous Injection of Niacin in Normal and Pyridoxine-Deficient Rats*—This experiment was conducted to determine whether pyridoxine deficiency has any influence on the synthesis of pyridine nucleotides from niacin by the erythrocytes in the rat. Since Handler and Kohn (7) found that free niacin was more effective than niacinamide for the coenzyme synthesis by human erythrocytes, the free vitamin was used in this investigation. 1 ml. of a sterile solution containing 10 mg. of nicotinic acid was injected subcutaneously into each of the normal and pyridoxine-deficient rats. The pyridine nucleotides in the erythrocytes were determined with the washed red blood cell suspension as described above. The results of both normal and the deficient groups are shown in Fig. 1. It will be seen that in both instances the response to niacin injection came earlier than after injection of tryptophan to normal rats, or after injection of pyridoxine and tryptophan to the deficient rats.

*Urinary Excretion of N<sup>1</sup>-Methylnicotinamide*—The excretion of this metabolite was first determined on a group of young rats after 7 weeks on the deficient diet. The average 24 hour excretion as measured from a 4 day collection of urine ranged between 17.2 to 39.4  $\gamma$  for the five pyridoxine-deficient rats, and from 43.5 to 510  $\gamma$  for the three controls. The intravenous injection of 50 mg. of L-tryptophan in the control animals produced an increase in excretion to 328  $\gamma$  per 24 hours compared to 92  $\gamma$  prior to injection. Deficient animals given 1 mg. of pyridoxine before the tryptophan injection excreted 421  $\gamma$  per 24 hours compared to 71  $\gamma$  previous to the injection. The subcutaneous injection of 10 mg. of niacin caused the excretion of N<sup>1</sup>-methylnicotinamide to increase from 93 to 595  $\gamma$  per 24 hours

in the control animals compared to an increase from 71 to 1193  $\gamma$  in pyridoxine-deficient animals. These figures are the average obtained with three or four animals in each case.

*Xanthurenic Acid Excretion in Pyridoxine Deficiency*—The xanthurenic acid excretion by three pyridoxine-deficient rats with an average daily food intake of 5.0, 3.5, and 8.5 gm. each was 0.59, 0.66, and 1.08 mg. respectively per 24 hours. The ration contained 25 per cent casein which yields 1.3 to 1.4 per cent of tryptophan (12, 13) and would provide approximately 17, 12, and 30 mg. of L-tryptophan respectively per day. When 150 mg. of DL-tryptophan along with 3 gm. of the ration were fed to each one of these rats, they excreted 13.44, 3.52, and 15.40 mg. of xanthurenic acid respectively per 24 hours. The excretion ceased abruptly after subcutaneous injection of 1 mg. of pyridoxine, but reappeared 48 hours later in one of the animals which excreted only 3.52 mg. in the first 24 hours after feeding tryptophan.

#### DISCUSSION

The present investigation reveals that intravenous injection of 50 mg. of L-tryptophan to normal rats induced a significant rise in total pyridine nucleotides in the erythrocytes which reached a peak 8 to 10 hours after the injection. The average maximal increase at 8 hours after the injection was 23 per cent over the initial levels. Calculated from the average weight of the rats and assuming their blood volume to be 6.4 per cent (6.2 to 6.7 per cent)<sup>1</sup> (14-16) and the average hematocrit at 8 hours to be 34.5 per cent, an erythrocyte volume of 7.1 ml. is obtained. This would account for a maximal increase in the erythrocytes of 230.8  $\gamma$  as DPN or 46.2  $\gamma$  as niacin per rat. It is reasonable to suppose that pyridine nucleotides are taken up by the tissues as they are synthesized, partly stored, and partly utilized in the metabolic processes, and then excreted as metabolites. Also a considerable portion of the converted niacin may be excreted in the urine as the free vitamin (3). Hence, no estimate of the amount of the injected tryptophan being converted into niacin can be made.

In the pyridoxine-deficient rats, intravenous injection of tryptophan caused no increment in the pyridine nucleotide content of the erythrocytes. The difference between the normal and the pyridoxine-deficient groups of animals 8 hours after the injection was  $43.6 \pm 16.2 \gamma$  as DPN per ml. of erythrocytes. The difference was more than twice the sum of the standard errors and was, therefore, statistically significant. Similar results were obtained by Rosen (4) and by Schweigert (3) and their respective coworkers by the measurement of urinary excretion of N<sup>1</sup>-methylnicotinamide and ni-

<sup>1</sup> Our own data, 6.6 per cent of the body weight determined with Evans blue (T-1824).

acin after the feeding or injection of tryptophan. Handler (17) suggested that "this defect in the metabolism of tryptophane in pyridoxine-deficient rats may be related to the demonstrated functioning of pyridoxine derivatives in decarboxylase and transaminase systems" as indicated by the investigations of Bellamy (18), Gunsalus (19), Umbreit (20), Schlenk and Snell (21), Lichstein (22), and their respective coworkers.

However, subcutaneous injection of 1 mg. of pyridoxine 1 hour prior to the injection of tryptophan to pyridoxine-deficient rats gave results similar to those found in normal animals. The highest increase in pyridine nucleotide content of the erythrocytes was 28.6 per cent over the average initial level. The erythrocyte volume calculated from the average weight and the hematocrit at 8 hours after the injection (42.3 per cent) was 6.0 ml. This would give a total maximal increment in the erythrocytes of the whole rat of 230.4  $\gamma$  as DPN, or of 46.0  $\gamma$  as niacin as against the 230.8  $\gamma$  as DPN, or 46.2  $\gamma$  as niacin found in the normal rats. Although the normal and the deficient animals differ considerably in their average weight and erythrocyte volume, the calculated average maximal increase per rat after the injection of tryptophan was almost equivalent in both groups when 1 mg. of pyridoxine was given to the deficient ones. These results suggest that the injection of pyridoxine to the deficient rats immediately restored their ability to convert tryptophan into niacin derivatives.

Anderson (23), Axelrod (24, 25), Dann (26), Kohn (27, 28), and their respective collaborators have found that, while factor V (coenzyme I) and the tissue niacin levels in muscle, liver, etc., decreased following the diminished intake of niacin in the diet, the coenzyme content in the erythrocytes was very little affected by the lowered intake. This was true with man and various species of animals, dogs, swine, chicks, etc. In the present investigation, it was found that the pyridoxine-deficient rats had a low pyridine nucleotide content of the erythrocytes. Therefore, it may not be amiss to presume that the pyridoxine-deficient rats suffer even greater privation of the coenzymes and niacin derivatives in the tissues than niacin-deficient animals. The findings of Rosen *et al.* (4) of a continued low urinary excretion of N<sup>1</sup>-methylnicotinamide long after refeeding pyridoxine to the deficient animals may be explainable in that the niacin derivatives formed from tryptophan were, to a great extent, synthesized into pyridine nucleotides and distributed in the tissues and the blood to restore and maintain their normal levels rather than excreted in the urine. The rapid growth and recovery of body weight after refeeding with pyridoxine cause swift gains in the masses of body tissues, and would further increase the storage of niacin derivatives and tryptophan in the body. These factors make it understandable that as long as the deficient animals are gaining in weight after restoration of pyridoxine to their diet much smaller amounts of metabolites

of niacin are excreted than by normal rats fed equal quantities of tryptophan.

The prompt responses of normal rats to the injection of tryptophan and the similar responses by pyridoxine-deficient animals to the combined injection of pyridoxine and tryptophan make it seem unlikely that intestinal flora are concerned in this conversion.

Subcutaneous injection of 10 mg. of niacin to normal and pyridoxine-deficient rats induced about the same amount of nucleotide synthesis by the erythrocytes and the excretion of large quantities of N<sup>1</sup>-methylnicotinamide in the urine in both groups. There was no significant difference in the reaction to the injection of niacin in normal and deficient animals. Hence, it may be inferred that pyridoxine derivatives are not involved in the synthesis of pyridine nucleotides from niacin.

The injection of 10 mg. of niacin resulted in a smaller increment in the pyridine nucleotides in the erythrocytes, but a greater urinary excretion of N<sup>1</sup>-methylnicotinamide than was found after intravenous injection of 50 mg. of L-tryptophan. These facts suggest that the injected vitamin was quickly removed from the blood stream and excreted in the urine as the methylated product, whereas the niacin derivatives formed in the body from tryptophan were better utilized for synthesis of coenzyme and a higher percentage of it was preserved in the animal body. Approximately from 8 to 27 per cent of the injected niacin was recovered from the urine as N<sup>1</sup>-methylnicotinamide in the next 48 hours following the injection. This agrees with the findings of Rosen, Huff, and Perlzweig (4). Schweigert and Pearson (3) found that a considerable portion of niacin was excreted as the free vitamin. Knox and Grossman (29) reported that in man about 10 per cent of the administered nicotinamide was isolated from the urine as 6-pyridone.

The urinary excretion of N<sup>1</sup>-methylnicotinamide after the injection of 50 mg. of L-tryptophan did not increase as much as when 100 mg. of DL-tryptophan were fed orally to the rats by Rosen *et al.* (4). Schweigert and his coworkers (30) pointed out that tryptophan is rapidly removed from the blood of both normal and B<sub>6</sub>-deficient rats after injection and, therefore, less niacin was formed than when the same amount of the amino acid was fed to the animals.

#### SUMMARY

1. The changes of total pyridine nucleotide levels in the erythrocytes after intravenous injection of 50 mg. of L-tryptophan to normal and pyridoxine-deficient rats showed that there was a significant rise in normal rats but no increase when the same injection was made to pyridoxine-deficient animals.

2. Subcutaneous injection of 1 mg. of pyridoxine hydrochloride to pyridoxine-deficient rats 1 hour prior to the injection of tryptophan incited prompt response and the increment in the pyridine nucleotide content of the erythrocytes was the same as in normal rats. This indicates immediate recovery of the animal's ability to convert tryptophan into niacin derivatives.

3. The response to intravenous tryptophan occurred as early as 4 hours and reached its peak from 8 to 10 hours after the injection, suggesting that bacterial synthesis in the intestine is not concerned in this conversion.

4. In contrast to the results after tryptophan injection, normal and pyridoxine-deficient rats showed no significant difference in pyridine nucleotide synthesis by the erythrocytes after injection of niacin. Hence, it may be deduced that pyridoxine derivatives are not involved in the synthesis of pyridine nucleotide from niacin.

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# THE DECARBOXYLATION OF L-PHENYLALANINE BY STREPTOCOCCUS FAECALIS R\*

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Gale (1) and Epps (2) have reported the properties of an enzyme obtained from various strains of *Streptococcus faecalis* which decarboxylates tyrosine and dihydroxyphenylalanine. Both of these authors reported that the enzyme preparations, whether made by washing the whole organism, by drying the cells with acetone, or by further purification procedures, were specific to the above two amino acids.

In the course of studies in which we were employing an acetone powder of *Streptococcus faecalis* R as a reagent for the estimation of tyrosine in the presence of other amino acids, we noticed that under certain conditions the medium apparently contained more tyrosine than the amount originally added. Upon investigation, this proved to be due to a concomitant decarboxylation of phenylalanine by the decarboxylase preparation.

The culture of the organism and the preparation of the acetone powder have been previously described (2). We employed a medium containing 30 gm. of casein acid hydrolysate (Stearns, amino acids 20 per cent solution), 10 gm. of glucose, and 5 gm. of dried yeast extract (Difco) per liter, sufficient dipotassium phosphate being added to bring the pH up to 6.0. The organism was grown at 37° for 16 hours.

All measurements were made with the Warburg apparatus at 38° with nitrogen as the gas phase, the acetone powder being added from the side arm as a suspension in 0.3 M citrate buffer, pH 5.4. The main compartment contained the substrates in the same buffer. The L-phenylalanine employed gave no color with the Millon's test for tyrosine.

Fig. 1 illustrates the decarboxylation of 16 micromoles of L-phenylalanine by 50 mg. of the acetone powder. The reaction is 98 per cent completed in 6 hours under these conditions. In similar experiments, 60 mg. of the enzyme preparation caused 100 per cent decarboxylation of the amino acid in the same time.

We could demonstrate no effect of the presence of L-phenylalanine on the decarboxylation of L-tyrosine by the preparation (Fig. 2), even though 40 micromoles of L-phenylalanine were added as compared to 5 micromoles

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of tyrosine. The low activity with phenylalanine, approximately 0.01 that obtained with tyrosine, coupled with the inability to saturate the enzyme with tyrosine because of the latter's insolubility, makes it difficult to show whether the two reactions are additive.

Since our purpose was not to attempt to embellish the excellent kinetic studies of Gale and Epps, but rather to point out a source of error in employing the enzyme preparation in the specific analysis of tyrosine, we have not pursued the purification further, and cannot say whether tyrosine decar-

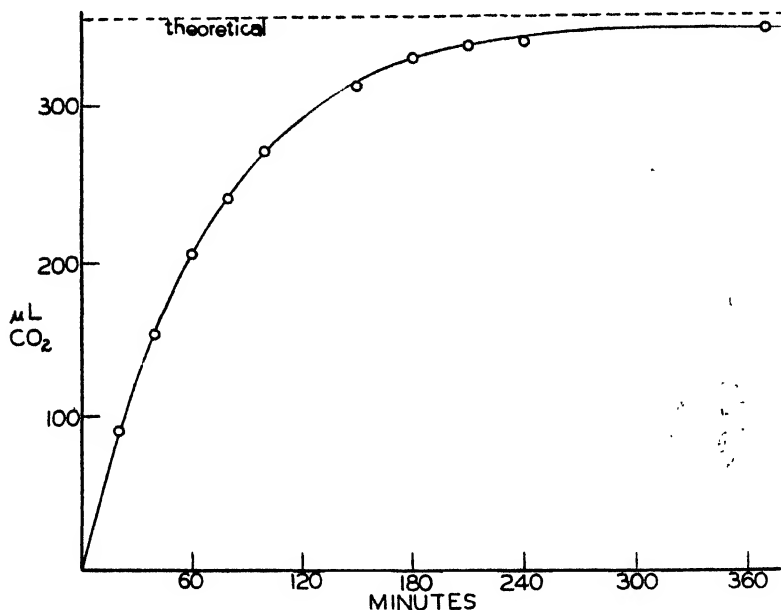


FIG. 1. Decarboxylation of L-phenylalanine. The main compartment contained 16 micromoles of L-phenylalanine in 2.5 ml. of 0.3 M citrate buffer at pH 5.4. 50 mg. of acetone powder suspended in 0.5 ml. of citrate buffer were added from the side arm at zero time; incubated at 38° with nitrogen as the gas phase.

boxylase also decarboxylates phenylalanine, or whether a second decarboxylase is present in the acetone powder. In an experiment in which 50 mg. of the acetone powder produced 218 microliters of CO<sub>2</sub> in 1 hour from L-phenylalanine, no decarboxylation of L-alanine, L-tryptophan, or L-proline could be demonstrated.

In order to identify the end-product of the reaction, 1.0 gm. of DL-phenylalanine and 2.0 gm. of acetone powder were incubated with 500 ml. of 0.3 M citrate, pH 5.4, at 38° for 7 hours. The mixture was made alkaline by the addition of 50 ml. of 50 per cent NaOH, and extracted with three 250 ml. portions of ethyl ether. The ether was separated, washed with 100 ml.

of water, and dried 3 days over anhydrous sodium sulfate. The ether was removed and the salt washed with anhydrous ether. The combined extract and washings were evaporated *in vacuo*, yielding a viscous brown oil, weighing 207 mg. The oil was treated with excess benzoyl chloride in alkali in the usual manner, and the solid which separated was filtered off

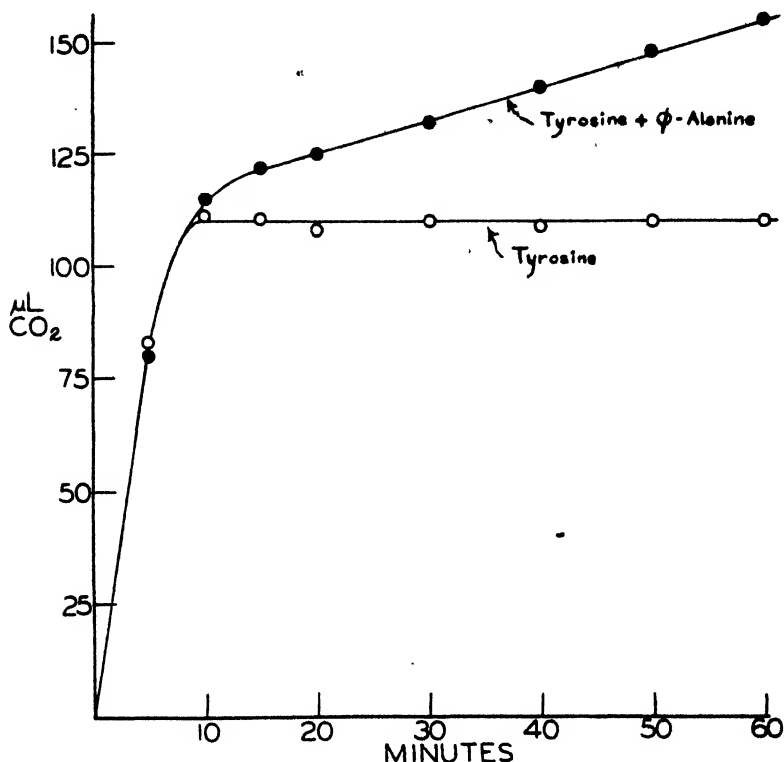


FIG. 2. Decarboxylation of L-tyrosine in the presence of L-phenylalanine. O, the main compartment contained 5 micromoles of L-tyrosine in 3.0 ml. of 0.3 M citrate buffer, pH 5.4. ●, the main compartment contained 5 micromoles of tyrosine and 40 micromoles of L-phenylalanine in 3.0 ml. of the citrate buffer. 5 mg. of acetone powder suspended in 0.5 ml. of citrate buffer were added from the side arm at zero time; incubated at 38° with nitrogen as the gas phase.

and washed with alkali and with water. It was recrystallized from an alcohol-water mixture, yielding 195 mg. of white crystals, melting at 114–115°. The benzoyl derivative was hydrolyzed in boiling 2 N NaOH, the hydrolysate extracted with ether, and the ether removed by distillation. The resultant oil was then treated with excess benzene sulfonyl chloride, the insoluble sodium salt precipitating. The reaction mixture was made

acid, and extracted with ether. The ether was evaporated, and the resultant oil recrystallized twice from alcohol-water mixtures, the initial impure yellow crop of crystals (m.p. 60–63°) being discarded. The once recrystallized second crop of transparent leaflets melted at 64.5–65.5°, which was raised to 66–66.5° upon the second recrystallization. The two products are evidently N-( $\beta$ -phenylethyl)benzamide and N-( $\beta$ -phenylethyl)benzenesulfonamide, respectively.

#### SUMMARY

The decarboxylation of L-phenylalanine by an acetone powder of *Streptococcus faecalis* R is reported.

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# THE UTILIZATION OF VITAMIN A IN VARIOUS CARRIERS

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At present, the vitamin A content of oils is evaluated by spectrophotometric methods. To ascertain whether the vitamin oil has been oxidized to any extent, the extinction coefficient is determined at 300 m $\mu$  on the slope of the vitamin A absorption curve as well as at the absorption maximum at 328 m $\mu$ . The ratio of these two values,  $E_{1\text{cm}}^{1\%}$  300:328 m $\mu$  (1), is taken as an index of the oil quality, 0.73 being generally accepted as the maximum ratio which a good oil may have (2, 3). Halpern (4) found that the increase of the ratio  $E_{1\text{cm}}^{1\%}$  300:328 m $\mu$  is due to the formation of a new maximum at about 280 m $\mu$  which steadily increases during the oxidation of vitamin A oils. The ratio  $E_{1\text{cm}}^{1\%}$  280:328 m $\mu$  is more indicative of oxidative changes in vitamin A oils than is the ratio  $E_{1\text{cm}}^{1\%}$  300:328 m $\mu$ .

Fridericia (5) found in 1924 that lard heated in thin layers and then, along with butter fat, fed to rats would destroy the vitamin A in the butter fat. Later, this observation was confirmed by many investigators. Lease *et al.* (6) used the liver storage technique in rats to determine the action of rancid fats upon the action of vitamin A. They found that when the peroxide number of lard was increased to 4 less vitamin A was stored, and at values of 11 or 30 only small amounts were retained in the liver. In general, the destructive action increased with the peroxide number. Dyme *et al.* (7) obtained similar results. They attributed the destructive action of rancid fats not only to peroxides but also to other decomposition products of fats.

The state of dispersion in which vitamin A is fed also affects its utilization. Halpern and Biely (8) found that vitamin A is better utilized in chicks when fed as a water emulsion than in a vegetable oil carrier. Likewise Sobel *et al.* (8) made the same observation with rats in tests on vitamin A storage in liver. A similar finding was reported on feeding carotene to rats (9).

The present study was carried out to investigate the influence of hydroperoxides and of the spectrophotometric extinction ratios of vitamin A oils upon the utilization of vitamin A in chicks. Vitamin A oils with different

spectrophotometric extinction ratios were investigated. They were dissolved in various vegetable oils with various peroxide numbers and also dispersed in water to form emulsions. Two series of biological experiments were carried out, one with New Hampshire pullet chicks, the other with white Leghorn cockerel chicks.

## EXPERIMENTAL

*Preparation of Vitamin A Oils*—Grayfish liver oils of about 8000 to 10,000 units of vitamin A per gm. were selected for the experiments. Five different oils were used during Series 1 and 2 of feeding experiments. Their

TABLE I  
*Physicochemical Characteristics of Grayfish Liver Oils*

	Oil 1	Oil 2	Oil 3	Oil 4	Oil 5
Vitamin A estimate at 328 m $\mu$ on whole oil, <i>units</i>	8160	9000	8300	8020	10,115
Ratio $E_{1\text{ cm.}}^{1\%}$ 260:328 m $\mu$ . . . . .	0.326	0.738	0.328	0.383	0.533
" " 280:328 " . . . . .	0.420	0.807	0.422	0.474	0.613
" " 300:328 " . . . . .	0.690	0.883	0.691	0.690	0.770
" " 350:328 " . . . . .	0.574	0.565	0.570	0.578	0.570
Vitamin A estimate at 325 m $\mu$ on unsaponifiable fraction, <i>units</i>	8330	8271	8330	7955	9790
Ratio $E_{1\text{ cm.}}^{1\%}$ 260:325 m $\mu$ . . . . .	0.236	0.330	0.230	0.265	0.262
" " 280:325 " . . . . .	0.347	0.446	0.358	0.362	0.367
" " 300:325 " . . . . .	0.656	0.728	0.659	0.681	0.674
" " 350:325 " . . . . .	0.466	0.478	0.480	0.498	0.493
Peroxide value . . . . .	0.0	16.7	0.8	8.0	27.5
Free fatty acid, % . . . . .	0.23	0.67	0.30	0.60	0.92

physicochemical characteristics are listed in Table I. Of the oils used for the first experiment, Oil 1 was freshly prepared from selected fresh livers, and Oil 2 was blended from two grayfish liver oils which had undergone considerable oxidation during long storage. Oils 3, 4, and 5 were used in Series 2 of feeding experiments. Oil 3 was the freshly prepared grayfish liver oil used in Series 1. During the 5 months between the experiments it was kept deaerated and stabilized at  $-25^{\circ}$ . The spectrophotometric characteristics of this oil did not change significantly during this period. Grayfish liver Oils 4 and 5 were prepared from commercial oils by bubbling air at about  $85^{\circ}$  until the desired peroxide values were reached. A comparatively high peroxide value can be obtained by this method without affecting the spectrophotometric values. As control oil, reference cod liver oil was used in Series 2.

Before blending, all oils were deaerated in a vacuum, saturated with carbon dioxide, stabilized with 0.05 per cent mixed tocopherols, and stored under nitrogen. Oils 1 and 2 contained in addition 0.1 per cent soy bean lecithin.

The spectrophotometric determinations were carried out with a Beckman spectrophotometer. The analyses on the unsaponifiable fraction were made by using essentially the method of Oser *et al.* (1). The isopropanol used in the procedure was purified by refluxing with NaOH and zinc dust, and was subsequently distilled. The iodometric determinations of peroxide values were made according to the method of Lea (10).

Vitamin A estimates carried out on the unsaponifiable fraction were used in preparing the blends. In this way the absorption of the saponifiable portion and of a part of the oxidized vitamin A was eliminated (4). Moreover, it was found by Halpern (4) that the absorption of the unsaponifiable fraction apart from vitamin A remains fairly constant during oxidation of vitamin A oils of high potency. The potency estimates of fresh grayfish liver oils were approximately equal on the whole oil and on the unsaponifiable fraction. The difference, however, increases with oxidized oils because of increased absorption of the saponifiable fraction.

The potency of grayfish liver Oil 1 was found to be higher on the unsaponifiable fraction than on the whole oil. The analyses were repeated three times in duplicate, always with the same result. It must be assumed, therefore, that this particular grayfish liver oil contained substances which slightly depressed the absorption at  $328\text{ m}\mu$  on the whole oil. The difference did not appear in Oil 3, probably because of a slight increase in extraneous absorption at  $328\text{ m}\mu$  on the whole oil during storage at  $-25^\circ$ .

*Preparation of Vegetable Oils*—In Series 1 of feeding experiments fresh and oxidized sunflower seed oils were used (Table II). The fresh oil was a commercial product with a peroxide value of 2.75; the oxidized oil was an old oil with a peroxide value of 9.0, and for the purpose of the experiment it was further oxidized by bubbling air at  $90^\circ$  until a peroxide value of 17.9 was reached. In Series 2 three different cottonseed oils were used. The fresh oil was very carefully selected, but it was not possible to obtain an oil free from peroxides. Oil 3 was a freshly refined cottonseed oil. Cottonseed Oils 4 and 5 were commercial oils oxidized by bubbling air at  $90^\circ$ . All vegetable oils were stabilized with 0.05 per cent of mixed tocopherols, deaerated in a vacuum, saturated with  $\text{CO}_2$ , and stored under nitrogen.

*Preparation of Blends*—The oils were blended under an inert gas to the desired potencies. The water emulsions for Series 1 were prepared without any fatty emulsifying agent. A 1 per cent solution of methocel (400 centipoise-Dow methyl cellulose, pharmaceutical grade) was used. The methocel solution was deaerated and saturated with carbon dioxide; 0.05 per cent

of gallic acid was added as antioxidant, and the solution was blended with the calculated amount of grayfish liver oil in a Waring blender under an inert gas. The emulsions were fairly stable when kept in a cool place.

TABLE II  
*Peroxide Values and Per Cent Free Fatty Acid of Vegetable Oils*

Oil No.	Oil	Peroxide value	Free fatty acid
			<i>per cent</i>
1	Sunflower seed oil, commercial product	2.75	0.3
2	" " " old, oxidized	17.90	0.5
3	Cottonseed oil, freshly refined	2.00	0.1
4	" " commercial, oxidized	9.10	0.6
5	" " " "	45.00	0.4

TABLE III  
*Results of Series 1 of Feeding Experiments*

Group No.	Fed 1 cc. every 2nd day	Description	Growth of chicks				Total mortality
			37 days	44 days	51 days	Average of Groups a and b, 51 days	
	<i>units per cc.</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
1a	50	Vitamin Oil 1	326.0	383.9	400.1	474.7	1
1b	150	In vegetable Oil 1	408.6	507.4	549.3		1
2a	50	Vitamin Oil 1	281.3	314.8	336.0	413.5	7
2b	150	In vegetable Oil 2	372.7	458.7	491.0		1
3a	50	Vitamin Oil 2	289.6	327.1	349.5	422.7	5
3b	150	In vegetable Oil 1	377.0	461.6	495.9		0
4a	50	Vitamin Oil 2	297.2	365.3	366.3	416.4	6
4b	150	In vegetable Oil 2	365.0	449.2	466.6		2
5a	50	Vitamin Oil 1	378.4	448.9	467.6	541.8	0
5b	150	In water emulsion	451.0	578.5	616.1		0
6a	50	Vitamin Oil 2	325.9	400.3	416.9	496.1	0
6b	150	In water emulsion	407.5	534.6	575.4		0
Control	0	No vitamin A	All dead				15

In order to improve the stability of the emulsions employed in Series 2, Dermal 14 (Emulsol Corporation) was used in addition to methyl cellulose.

TABLE IV  
Results of Series 2 of Feeding Experiments

Group No.	Fed 1 cc. every 3rd day	Description	Growth of chicks			Total mortality
			35 days	42 days	Average of Groups a and b, 42 days	
	<i>units per cc.</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
1a	75	Reference cod liver oil	326.3	400.7	450.6	0
1b	225	In vegetable Oil 3	388.2	500.5		0
2a	75	Vitamin Oil 3	347.5	428.1	458.7	0
2b	225	In vegetable Oil 3	374.6	489.2		0
3a	75	Vitamin Oil 4	332.1	396.5	441.0	0
3b	225	In vegetable Oil 3	370.5	485.5		0
4a	75	Vitamin Oil 5	335.7	396.1	441.3	1
4b	225	In vegetable Oil 3	373.9	486.5		1
5a	75	Vitamin Oil 3	371.4	465.5	488.9	0
5b	225	In emulsion	378.4	512.3		0
6a	75	Vitamin Oil 4	374.5	475.9	494.1	1
6b	225	In emulsion	374.9	512.3		0
7a	75	Vitamin Oil 5	368.0	471.2	493.7	0
7b	225	In emulsion	391.2	516.1		0
8a	75	Vitamin Oil 3	337.9	427.9	456.5	0
8b	225	In vegetable Oil 4	367.6	485.0		0
9a	75	Vitamin Oil 3	349.7	425.1	450.8	0
9b	225	In vegetable Oil 5	359.5	476.4		0
10a	75	Vitamin Oil 5	338.0	432.3	451.4	1
10b	225	In vegetable Oil 4	352.6	470.4		0
11a	75	Vitamin Oil 5	320.6	398.3	426.3	3
11b	225	In vegetable Oil 5	355.6	454.3		0
12a	0	No vitamin A	257.9	All dead		16
12b	0	" " "	241.0	258.3		12

Previously it was found that Demal 14 does not change the stability of gray-fish liver oils toward oxidation. Mixed tocopherols were added to all emulsions so that the total tocopherol content per cc. was equal to the tocopherol



content per cc. of the vitamin solutions in oil. In this way possible variations of biological activity due to the action of tocopherols were eliminated (11, 12).

*Chick Tests*—Two series of biological tests with growing chicks were carried out at an interval of 5 months.

In Series 1 New Hampshire day-old pullet chicks were depleted on a vi-

TABLE V  
*Analysis of Variance of Final Weights of Chicks, Series 1*

Variation source	Sum of squares	Degrees of freedom	Mean square	Minimum significant difference	
				5 per cent	1 per cent
Total.....	1,508,878	131	11,518		
Levels.....	618,682	1	618,682*	24	31
Treatments.....	265,386	5	53,077*	41	54
Interactions.....	58,752	5	11,750†	58	
Error.....	566,058	120	4,717		

\* Highly significant.

† Significant.

TABLE VI  
*Analysis of Variance of Final Weights of Chicks, Series 2*

Variation source	Sum of squares	Degrees of freedom	Mean square	Minimum significant difference	
				5 per cent	1 per cent
Total.....	1,398,929	327	4,236		
Levels.....	307,475	1	307,475*	12	16
Treatments.....	154,251	10	15,425*	28	36
Interactions.....	37,664	10	3,766		
Error.....	899,539	306	2,940		

\* Highly significant.

tamin A-deficient diet for 7 days and then distributed into nine lots of fifteen chicks. The average weights of the lots were approximately equal.

The chicks were reared in Jamesway battery brooders until they reached 5 weeks of age and were then transferred to holding battery brooders. Throughout the experiment the chicks had free access to the vitamin A-deficient diet and water. The chicks were fed the vitamin A preparations every other day by mouth with pipettes calibrated to deliver 1 cc. To equalize the fat intake in all lots, the chicks that were fed water emulsions of vitamin A received also 1 cc. of sunflower seed oil on days when they were not fed vitamin A.

Series 2 was carried out with white Leghorn day-old cockerel chicks under conditions similar to those of Series 1. The chicks were depleted on a vitamin A-deficient diet and standardized as to weight at 14 days of age. Fifteen chicks were placed in each lot and fed the vitamin A preparations by mouth with pipette every 3rd day. As in Series 1 the fat intake of the chicks receiving the water emulsions was equalized by feeding 1 cc. of fresh cottonseed oil. This experiment was discontinued 1 week earlier than planned owing to the fact that several lots of chicks became infected with coccidiosis after being transferred to the holding batteries.

The chicks in both experiments were weighed at weekly intervals and a record was kept of symptoms of disease and mortality. Mortality, except in a few lots fed oxidized oils, was practically nil (Tables III and IV).

In both experiments the chicks received the equivalent of 25 and 75 units of vitamin A daily. These levels of vitamin A were chosen because previous experience (13) showed that at these levels chicks would show significant differences in weight as well as low mortality. Some of the chicks in the control lots lived to 6 weeks of age, even though they showed such advanced symptoms of vitamin A deficiency as staggering gait, ruffled feathers, swollen eyes, and slight beak necrosis. The average weights of chicks in Series 1 and 2 together with other pertinent data are summarized in Tables III and IV. The final weights were analyzed statistically and the essential data are given in Tables V and VI.

#### DISCUSSION

*Grayfish Liver Oils*—From the data presented in Tables I, III, and IV, it is apparent that the biological value of the grayfish liver oils depended mainly on the spectrophotometric characteristics of the oils. A critical examination of the extinction ratios shows that Oil 2 was the only one with an  $E_{1\text{cm}}^{1\%}$ , 280:325  $m\mu$  ratio of over 0.4 for the unsaponifiable fraction. Only this grayfish liver oil produced a considerably depressed growth in vegetable oil as well as in emulsion. The ratio of 0.4 for  $E_{1\text{cm}}^{1\%}$ , 280:325  $m\mu$  characterizes a highly oxidized grayfish liver oil (4) and possibly represents the limit at which the vitamin A of grayfish liver oil can be fully utilized by the chick. The extinction ratios taken at 300  $m\mu$  showed on all oil samples a similar trend to the ratios at 280  $m\mu$ . For Oil 2 the  $E_{1\text{cm}}^{1\%}$ , 300:325  $m\mu$  ratio of 0.728 was, however, a border line value (5) and did not indicate so clearly as the 280  $m\mu$  ratio the decreased biological action of the oil.

The whole oil ratios at 280 and 300  $m\mu$  both showed the same trend but did not appear to be so indicative of the quality of the grayfish liver oils as were the ratios taken on the unsaponifiable fraction. For example, Oil 5 had abnormally high ratios on the whole oil but produced a growth rate equal to that obtained with fresh Oil 3.

The rôle of the hydroperoxides in the grayfish liver oils was found to be insignificant. Considering the extent to which the grayfish liver oils were diluted for the feeding experiments, this could rather be expected. The grayfish liver oils dissolved in fresh vegetable oil and in water emulsion showed biological values which were independent of their hydroperoxide content. It can be seen from Tables I and IV that grayfish liver Oils 4 and 5 with peroxide values of 8.0 and 27.5 respectively did not produce significantly different growth from fresh grayfish liver Oil 3 with a peroxide value of 0.8. Grayfish liver Oil 2, however, with a peroxide value of 16.7 in Series 1, produced a significantly lower growth response than fresh Oil 1 when fed in vegetable oil as well as when fed in emulsion. In explanation of the apparent discrepancies in the activity of grayfish liver Oil 2 in the Series 1 and grayfish liver Oils 4 and 5 in Series 2, it will be noted that grayfish liver Oil 2 had considerably higher ratios on both the whole oil and the unsaponifiable fraction than any of the other grayfish liver oils including Oil 5, which had a substantially higher peroxide value.

*Vegetable Carrier Oils*—The amount of hydroperoxides fed through the medium of the vegetable carrier oils was naturally much larger than that from the grayfish liver oils. A more pronounced peroxide action would therefore be expected in the groups in which oxidized vegetable oils were fed as carriers.

In Group 2 of Series 1 (Table III), an old sunflower seed oil oxidized by prolonged storage and showing a peroxide value of 17.9 was used as a carrier for the fresh grayfish liver Oil 1. Its effect was to decrease significantly the biological action of the vitamin A. In Group 4 of Series 1 the same oxidized sunflower seed Oil 2 was fed as a carrier for oxidized grayfish liver Oil 2. The growth rate of the chicks did not decrease further. It can therefore be concluded that no cumulative action resulted from combining the oxidized vegetable oil with the oxidized grayfish liver oil, each of which oils alone decreased the biological action of vitamin A.

In the experiments of Series 2 vegetable Oils 4 and 5 were oxidized by bubbling air to peroxide values of 9.1 and 45.0 respectively. These two oils were compared to freshly refined vegetable Oil 3, which had a peroxide value of 2.0. It can be seen in Table IV, Groups 8, 9, and 10, that the oxidized oils did not depress the biological action of the grayfish liver oil. Only the growth rate of chicks in Group 11, in which both the grayfish liver oil and the vegetable oil were oxidized to high peroxide values, was slightly lower. The difference, however, was not statistically significant. This confirms the results obtained in Series 1 on the non-cumulative effect on vitamin A utilization of oxidized vegetable oil and oxidized grayfish liver oil when fed together.

It appears from the results of both series of experiments that the hydro-

peroxides alone did not inhibit the action of vitamin A in chicks, but that other substances were responsible for the decreased growth produced by vegetable Oil 2. These substances are probably decomposition products of the hydroperoxides.

It can be concluded from these results that the chick is more resistant toward the action of hydroperoxides than is the rat. As mentioned above, the destructive action of peroxides upon vitamin A has been established for the rat, the rate of destruction varying with the peroxide value (6). Furthermore, Smith (14) and Simmons *et al.* (15) found that peroxides also destroy vitamin A *in vitro*, the rate of destruction being again proportional to the peroxide value. The reaction rate *in vitro* is, however, much slower than *in vivo* where prooxidants act as catalysts.

*Emulsions*—In both feeding series, the vitamin A oils in emulsion showed significantly higher biological values than the same oils dissolved in fresh vegetable oil. In some of the treatments the differences in the rate of growth were statistically highly significant.

Both the fresh and the oxidized vitamin oils had significantly higher biological values when fed in emulsion. It appears that the improved action of vitamin A in water emulsion was not affected by the quality of the vitamin oil *per se*, but that the vitamin A was only better utilized. It was interesting to note that oxidized grayfish liver Oil 2 (Table III) in emulsion gave better growth response than fresh grayfish liver Oil 1 dissolved in fresh vegetable oil. The difference was not statistically significant, but it can be seen that even the highly oxidized vitamin Oil 2 in emulsion had at least the same value as fresh vitamin Oil 1 dissolved in fresh vegetable oil.

The better utilization of vitamin A in emulsion can be explained by the large surface between the oil and water phases. The large interfacial area provides for the rapid action of ester-splitting enzymes and, therefore, an accelerated formation and transfer of the vitamin A alcohol (16) by the lymphatic route from the danger zone in the intestinal lumen (17). The rate of destruction for vitamin A *in vivo* can be very rapid. Apart from the action of lipoxidase-salt systems, which could be responsible for the non-economic utilization of vitamin A, intestinal juice could be destructive of vitamin A, as was found for saliva, gastric juice (18), and bile (19).

#### SUMMARY

Grayfish liver oils having different spectrophotometric ratios and different peroxide values were dissolved (1) in vegetable oils of different peroxide values and (2) made into water emulsions. The vitamin A preparations were fed by mouth with pipette to chicks at the suboptimal rate of 25 and 75 units per day.

The growth response of the chicks to the various preparations was studied, and the following conclusions reached. (1) The spectrophotometric extinction ratios  $E_{1\text{cm}}^{1\%}$  280:325  $m\mu$  and 300:325  $m\mu$  were useful in evaluating the biological action of the grayfish liver oils. The ratio taken at 280  $m\mu$  was found to be more indicative of quality than the ratio at 300  $m\mu$ . (2) Hydroperoxides up to a peroxide value of 45.0 did not inhibit the action of vitamin A in chicks. Apparently other compounds than peroxides present in oxidized oils decreased the biological action of vitamin A. Furthermore, there was no cumulative inhibiting effect when an oxidized grayfish liver oil was fed in conjunction with an oxidized vegetable oil. (3) When fed orally, by pipette, vitamin A oils, whether fresh or oxidized, had a greater biological value in water emulsion than in vegetable oil solution.

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## REACTION OF FORMALDEHYDE WITH PROTEINS

### VI. CROSS-LINKING OF AMINO GROUPS WITH PHENOL, IMIDAZOLE, OR INDOLE GROUPS

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It has been recognized in recent years that, at elevated temperature or in acid or alkaline solution, formaldehyde can add as methylol groups to the amide (1, 2), guanidyl (3-8), and indole groups (9) of proteins. In contrast, the addition product of formaldehyde with amino groups forms instantaneously even at neutrality and low temperatures, but this is an equilibrium reaction and the methylol compound can be decomposed by dilution or dialysis of the reaction mixture (10). The loss of amino nitrogen observed in proteins that had been allowed to react with formaldehyde for several days and then isolated by dialysis (10-12, 7) thus required further explanation.

One mechanism has recently been proposed (13). Aminomethylol groups were found to condense with amide or guanidyl groups at room temperature and over the range of pH 3 to 8 to form comparatively stable compounds, whereas under the same conditions neither amines, amides, nor guanidyl compounds separately bound formaldehyde in a stable manner.

In the present paper it will be shown that similar cross-linking can also occur between aminomethylol groups and phenol, imidazole, or indole groups. The reactions involving amino groups, formaldehyde, and the reactive CH groups on the phenol or imidazole rings are typical of the Mannich reaction (14), which, although widely studied and used, has apparently not yet been considered in connection with protein-formaldehyde reactions.<sup>1</sup> Evidence has now been obtained that these reactions occur with proteins under conditions of pH and temperature which are used for tanning and for the preparation of toxoids and vaccines.

#### EXPERIMENTAL

The reaction conditions are indicated in the Tables I to VII. The reaction mixtures comprising only small molecular weight compounds were

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<sup>1</sup> Unlike the usual Mannich reaction (14), however, the present experiments were generally carried out with a great excess of formaldehyde in dilute aqueous solution.

subjected to analyses after suitable dilution. The pH of all reaction mixtures was measured, after dilution, by means of the glass electrode. Proteins, after reaction with formaldehyde, were isolated by dialysis (3 days against running tap water at room temperature and 3 days against distilled water at 3–5°). Control experiments showed that these conditions were amply sufficient for the removal of all free formaldehyde or other compounds added to the reaction mixtures. When necessary, the products were dried by lyophilization.

Gramicidin derivatives were isolated by precipitating the reaction mixture with about 10 volumes of 0.1 M sodium chloride, washing the precipitate with water or dilute saline solution, redissolving it with added ethanol, and again precipitating and washing it as before. Some derivatives, notably those containing proline or alanine, could be precipitated from 5 to 10 per cent alcoholic aqueous solution only by addition of sodium chloride to 0.3 M concentration. Many of the derivatives here described, in contrast to gramicidin or methylol gramicidin, yielded bulky gelatinous precipitates.

*Materials*—Gramicidin was kindly supplied by the Wallerstein Laboratories, isinglass by the Connaught Laboratories, and insulin by Eli Lilly and Company. The other proteins used have been previously characterized (1). Crystalline bovine serum albumin and all other chemicals were commercial preparations. The amino acids were DL preparations with the exception of L-histidine and L-tyrosine. The  $\alpha$ -N-acetyl derivatives of L-histidine, DL-tryptophan, and DL-tyrosine were prepared according to published methods (15–17). The acetylated proteins were prepared by selective reaction of the amino groups alone by means of acetic anhydride in an aqueous medium at 0° (18).

*Analytical Methods*—Free formaldehyde was determined with dimedon (19), but in the present study the combined determination of free plus reversibly bound formaldehyde was of greater value. This was achieved by a chromotropic acid colorimetric method (20), either directly on aliquots of the reaction mixture or, more generally, after combined acid hydrolysis and steam distillation (1). "Irreversibly bound formaldehyde" was the difference between the amount added to, and recoverable from, reaction mixtures. Controls indicated that there was no destruction of formaldehyde under the conditions of pH and temperature used for the experiments or for the analyses.

The amounts of formaldehyde bound irreversibly by model systems containing amines and phenol or imidazole derivatives supplied a valid means of gaging the extent of reaction. Colorimetric analyses yielded confirmatory evidence.

The participation of protein in similar reactions was primarily demonstrated through a study of their ability to fix small molecular weight

phenols, imidazoles, or indoles. The most suitable model compounds for this purpose were the corresponding  $\alpha$ -N-acetylamino acids, since the amount of acetyl groups thus introduced could be analytically determined after release by acid hydrolysis. To this end, 25 mg. samples, hydrolyzed with 1 ml. of 6 N sulfuric acid in sealed tubes at 100° for 18 hours, were subjected to vacuum distillation with repeated additions of water (total, 170 ml.) and titration of the distillate with 0.02 N sodium hydroxide, with phenolphthalein as indicator. When chlorophenols were used, chlorine analyses performed on the isolated proteins served as a measure of the extent of interaction. Colorimetric analyses supplied additional evidence.

Colorimetric determinations of the aromatic and heterocyclic compounds were performed for two different purposes. In the one case it was desired to demonstrate the involvement of these ring compounds in reactions with formaldehyde; in the other, to demonstrate the introduction of added simple phenols and indoles into proteins by means of the cross-linking methylene group. Different techniques were required. For the first purpose it was found that when phenols or imidazoles entered into condensation reactions their ability to combine with most color-forming reagents was considerably lowered. Thus decreases in chromogenic activity by the Thomas (21) or Macpherson (22) method indicated involvement of phenols and imidazoles, respectively.

With regard to histidine, and in confirmation of these observations, it was found that a derivative prepared from histidine and formaldehyde (23) according to Neuberger (24) (imidazole-tetrahydropyridine carboxylic acid, Compound B) gave a different color (yellow) and of a lower intensity (16 to 25 per cent) than an equimolar amount of histidine, both by the original Pauly method (25) and with the Macpherson modifications (22).<sup>2</sup> This reaction represents an intramolecular example of the same type of methylene condensation that probably occurs between amino and imidazole groups of proteins.

In order to demonstrate colorimetrically the introduction, through the Mannich reaction, of added simple phenols or indoles into proteins, a test was needed which would be positive for such compounds even after they entered into Mannich condensations. The Folin phenol reagent (26) was found to act in the desired manner. Actually increased chromogenic activities were obtained with Mannich derivatives of phenols, as compared to the original phenols. For the colorimetric detection of introduced histi-

<sup>2</sup> Neuberger (24) reported that the N-methylol derivative of Compound B has about the same chromogenic activity, on a molar basis, as does histidine. In other aspects of the reaction of histidine and formaldehyde, our observations are in accord with those of Neuberger, except that on several occasions Compound B was obtained as the monohydrate instead of the dihydrate.



dine derivatives, only the Macpherson method was used; because of the low chromogenic activity of Mannich derivatives of this amino acid, the resulting values when calculated from histidine standard curves were obviously much too low.

Proteins had to be hydrolyzed prior to application of the colorimetric tests. Methods of hydrolysis had to be such as not to reverse the Mannich reaction or to favor reactions between the liberated formaldehyde and cyclic amino acids. Indole-formaldehyde derivatives (9) and certain methylolphenols (27) are hydrolyzed by strong alkali, but the Mannich type of phenol-methylene derivatives proved to be stable under conditions of alkaline hydrolysis (5 hours at 120° with 5 N NaOH), which, in turn, did not favor secondary condensation reactions.

For histidine analyses acid hydrolysis was used (18 hours at 120° with 6 N HCl). When formaldehyde-treated proteins were hydrolyzed in this manner, reversibly bound formaldehyde was found to react with the liberated histidine (and tyrosine).<sup>3</sup> This could be prevented by the addition to the protein sample of about half its weight of dimedon, which served to trap the liberated formaldehyde. The hydrolysates were neutralized and centrifuged, but the usual separation of the histidine fraction was omitted, for fear that any formaldehyde condensation derivative of histidine might not appear quantitatively in the proper fraction. Thus tyrosine was not removed and must have contributed to the chromogenic activity of the hydrolysates. Control experiments showed, however, that under Macpherson's conditions, tyrosine gives only about 20 per cent of the color that is given by histidine, on a molar basis.

Acid and basic groups were determined by dye methods (28), amino nitrogen according to Van Slyke (29), and tryptophan according to Horn and Jones (30).

### *Results*

Two main types of experiments were performed in studying the Mannich interaction of aminomethylol groups with imidazole or phenolic groups. (a) The amount of formaldehyde bound irreversibly by model systems, comprising aliphatic amino acids and substituted imidazoles or phenols, was determined, and (b) simple phenols or acylhistidines were introduced through methylene linkage into proteins rich in amino groups. The latter techniques could be used also with indoles, but not the former, since simple indoles by themselves bind formaldehyde stably over a wide pH range (9),

<sup>3</sup> Histidine and tyrosine do not react with formaldehyde under the conditions of combined hydrolysis and steam distillation used in the formaldehyde analysis. Tryptophan and cysteine bind formaldehyde under these conditions (9).

in contrast to imidazoles or phenols (1, 24, 23).<sup>4</sup> Interaction of amines and substituted indoles through formaldehyde was further demonstrated with gramicidin, which contains 40 per cent of tryptophan.

*Model Systems*—Table I lists a series of experiments on the interaction of threonine, *p*-cresol, and formaldehyde at various levels of pH and tem-

TABLE I  
*Reaction between Threonine, p-Cresol, and Formaldehyde\**

	Final pH	Temperature °C.	Equivalent of formaldehyde bound	Apparent phenol†	
				Folin	Gerngross
Threonine + <i>p</i> -cresol.	3.9	23	1.0	1.7	0.7
“ + “	3.9	40	1.0	1.7	0.5
	4.0	23	0.0		
	3.9	40	0.1		
<i>p</i> -Cresol .....	4.1	40	0.2	1.0‡	1.0‡
Threonine + <i>p</i> -cresol.	5.5	40	0.9		0.5
	5.3	40	0.0		
<i>p</i> -Cresol .....	7.0	40	0.3	1.1	0.9
Threonine + <i>p</i> -cresol .....	6.8§	23	0.9	1.8	0.7
“ + “ .....	6.8§	40	1.0	1.8	0.5
	6.7§	23	0.0		
	6.8§	40	0.0		
<i>p</i> -Cresol	7.6§	23	0.2	1.0	1.0
	7.5§	40	0.5	1.1	0.8

\* 0.75 mm each of the amino acid and the phenol, the latter in 10 per cent solution in ethanol, treated for 7 days with 0.3 ml. of 3 M acetic acid or sodium acetate and 2.0 ml. of 7.5 per cent formaldehyde (5 mm).

† In terms of molar equivalents of untreated *p*-cresol. The latter gives only 55 per cent of the Folin color, but 140 per cent of the Gerngross color of an equimolar amount of tyrosine.

‡ The same chromogenic activity was observed with *p*-cresol which had been exposed for 7 days at 40° to acetate of pH 4.1 or phosphate of pH 7.6 in the absence of formaldehyde.

§ 3.4 M phosphate buffer (pH 7.6) was used instead of acetate.

perature. Threonine was chosen in preference to other amines or amino acids because it did not fix formaldehyde either by itself or in the presence of acetate buffers (13). *p*-Cresol alone also did not react appreciably at room temperature over the range of pH 4.1 to 7.6, nor at 40° up to pH 6.8.<sup>4</sup> When mixtures of equimolar amounts of threonine and *p*-cresol were treated

<sup>4</sup> Unpublished experiments of the authors have shown that *p*-cresol binds formaldehyde at an appreciable rate at pH 11.5, but not at pH 8.

with formaldehyde at room temperature, however, close to 1 equivalent was bound in acid-resistant manner over the entire pH range studied.

The chromogenic activities of the derivative of *p*-cresol in the reaction mixtures differed in a consistent manner from those of the untreated *p*-cresol. The Folin color (26) increased to almost double, while the Gerngross color (21) decreased to half. The observed loss in the Gerngross color was to be expected, since this reaction involves the carbon ortho to the phenolic group and thus generally serves as a means of differentiating ortho-substituted from unsubstituted phenols. It was therefore thought that 2,4-dimethylphenol, if it condensed with threonine and formaldehyde, should yield a di-ortho-substituted phenol, which might be expected to give no Gerngross color at all.

With this purpose in mind, 2,4-dimethylphenol was used as an additional model. While this phenol reacted somewhat more readily with formaldehyde alone than did *p*-cresol at pH 4 to 7 and room temperature or 40°, the reaction did not go beyond 20 per cent and 40 per cent, respectively, in 7 days (Table II). In contrast, in the presence of threonine complete reaction (*i.e.*, fixation of 1 equivalent of formaldehyde) was approached or reached, respectively, at the two temperatures. The condensation seemed to proceed slightly faster at pH 4.2 and 8.7 than at pH 5.6.

Colorimetric analyses showed that the Mannich condensation of this phenol was also accompanied by increases of the Folin color to almost double that of the starting material. However, as was expected, the Gerngross color disappeared as the reaction reached completion. This appears to be good evidence for the involvement of the ortho carbon atom in the reaction. Thus the doubling of one and the extinction of another chromogenic activity clearly characterize a Mannich reaction involving 2,4-dimethylphenol. These reactions also supplied useful evidence for the Mannich fixation of the dimethylphenol by proteins.

A series of experiments with model substances was performed in order to ascertain whether protein nitrogenous groups other than amino groups might undergo condensation with formaldehyde and phenols. In agreement with the literature (14), this appeared not to be possible. Neither amide nor guanidyl groups reacted with 2,4-dimethylphenol and formaldehyde during 1 week at 40° (Table III).

When similar model experiments were performed with threonine, formaldehyde, and  $\alpha$ -N-acetyl-L-histidine or L-histidine anhydride, condensation was found to occur at 40°, but not appreciably at room temperature. The results of a few illustrative experiments are summarized in Table IV. The fixation of formaldehyde proceeded furthest (84 per cent) at pH 6.4 with acetylhistidine, and at pH 5.0 (76 per cent) with histidine anhydride.

The extent of interaction was in each case less marked when estimated from the change in chromogenic activity than from formaldehyde fixation (as determined both by chromotropic acid after distillation and with

TABLE II  
*Reaction between Threonine, 2,4-Dimethylphenol, and Formaldehyde\**

	Final pH	Time	Temper- ature	Equiv- alents of formalde- hyde bound	Apparent phenol†	
					Folin	Gern- gross
		days	°C.			
Threonine + dimethylphenol . . .	4.2	2	23	0.6	1.5	0.5
+       "       . . .	4.2	7	23	0.8	1.7	0.2
+       "       . . .	4.2	7	40	1.1	1.9	0.0
	3.9	7	40	0.1		
Dimethylphenol . . . . .	4.2	7	23	0.2	1.0	1.1
	4.2	7	40	0.4	1.0	1.0
Threonine + dimethylphenol . . .	5.6	1	23	0.3	1.4	0.5
+       "       . . .	5.6	2	23	0.4	1.6	0.2
+       "       . . .	5.6	7	23	0.7	1.8	0.0
+       "       . . .	5.6	7	40	1.0	1.9	0.0
+       "       . . .	6.7‡	7	40	0.8	1.8	0.0
	5.3	7	40	0.0		
	6.7‡	7	23	0.0		
	6.8‡	7	40	0.0		
Dimethylphenol . . . . .	7.0	2	23	0.2	1.0	1.2
	7.0	7	23	0.3	0.9	1.1
	6.7	7	40	0.4	1.0	0.9
	7.6‡	7	40	0.2	1.0	0.7
Threonine + dimethylphenol . . .	8.7	1	23	0.5	1.0	0.2
+       "       . . .	8.7	2	23	0.7	1.2	0.1
	8.5	2	23	0.0		
Dimethylphenol . . . . .	8.8	2	23	0.2	1.0	0.8
Threonine + dimethylphenol . . .	9.9	2	23	0.6	1.6	0.0
Dimethylphenol . . . . .	9.8	2	23	0.4	1.1	0.1

\* 0.75 mm each of the amino acid and the phenol, the latter in 10 per cent solution in ethanol, treated with 0.3 ml. of acetic acid or sodium acetate, borate (pH 8 to 9) or hydroxide (pH 9.8 to 9.9), and 2.0 ml. of 7.5 per cent formaldehyde (5 mm).

† In terms of molar equivalents of untreated 2,4-dimethylphenol. The latter gives 69 and 109 per cent of the Folin and Gerngross color, respectively, of an equimolar amount of tyrosine.

‡ 3.4 M phosphate buffer (pH 7.6) was used instead of acetate.

dimedon). This can be due only in part to the residual chromogenic activity of the reaction product. A possible explanation might be that condensation involved primarily the imino group of the imidazole nucleus,

particularly near pH 4, and that only the secondary stable (*i.e.*, Mannich) fixation of the substituted methylene group by the reactive CH group led to a loss in chromogenic activity (*cf.* (24)).

A series of experiments with formaldehyde, acetylhistidine, and either proline, acetamide, or methylguanidine sulfate showed that, as in the case of phenols, condensation of imidazoles occurred only in the presence of amino or imino groups (Table IV).

*Introduction of Phenols, Indoles, or Imidazoles into Proteins through Formaldehyde*—The occurrence in proteins of the cross-linking condensation of formaldehyde, amino, and phenol or imidazole groups is strongly suggested

TABLE III

*Lack of Reaction between 2,4-Dimethylphenol, Formaldehyde, and Either Acetamide or Methylguanidine Sulfate\**

	Final pH	Equivalent of formaldehyde bound	Apparent phenol†	
			Folin	Gerngross
Methylguanidine + dimethylphenol...	4.2	0.2	1.0	1.1
“ + “ .....	6.1	0.3	1.0	1.0
Acetamide + dimethylphenol .....	4.2	0.3	1.0	1.0
“ + “ .....	6.3	0.3	1.0	1.0
Methylguanidine .....	6.2	0.1		
Acetamide .....	6.4	0.3		
Dimethylphenol .....	4.2	0.2	1.0	1.1

\* 1 week at 40°. Concentrations and buffers same as in Tables I and II.

† See foot-note to Table II.

by the above findings. However, no unequivocal technique for its demonstration appeared available.<sup>5</sup> Only the alteration of the chromogenic activity of the respective amino acid residues could be used as indication of their participation in Mannich reactions. The control data obtained with formaldehyde-treated serum albumin as compared to its aminoacetyl derivative represent such evidence of the occurrence of Mannich reactions (at 40°) with the phenol groups (Table V). More striking changes in the chromogenic properties of proteins were obtained at higher temperatures, but under such conditions the rôle of the amino groups became less clearly apparent.

It appeared that the reaction might be demonstrated unequivocally through the fixation, in the presence of formaldehyde, of small molecular

<sup>5</sup> Fixation of formaldehyde and loss of amino nitrogen can be due to condensation between amino and amide or guanidyl groups (13), in addition to the reactions discussed above.

weight phenols, imidazoles, or indoles by proteins rich in amino groups. As previously stated, the corresponding acetyl amino acids were chosen for

TABLE IV

*Reaction between Amino Acids, Acetyl-L-histidine or Histidine Anhydride, and Formaldehyde\**

Ex- per- iment No.		Final pH	Equivalents of formaldehyde bound		Apparent histidine†
			Irre- versibly	Total	
A*	Threonine + acetylhistidine	3.5	0.3	0.3	1.0
	“ + “	4.5	0.6		0.9
	“ + “	6.4	0.8	0.8	0.6
	“ + histidine anhydride	4.1	0.4		1.0
	“ + “ “	5.0	0.7	0.7	0.9
	“ + “ “	6.8	0.4	0.6	0.8
	“	3.9-6.8	0.0		
	Acetylhistidine	3.5	0.0		1.0
	Histidine anhydride	7.5	0.0		1.0
	Threonine + acetylhistidine	7.3	0.4		0.8
B*	“	7.3	0.0		
	Acetylhistidine	7.3	0.0		1.0
	Serine + acetylhistidine	7.3	0.8		0.6
	“	7.3	0.3		
	Acetylhistidine	7.3	0.0		1.0
	Threonine + acetylhistidine	6.6	1.1†		0.5
C‡	Alanine + “	6.7	1.8†		0.5
	Proline + “	6.8	1.4†		0.8
	Acetamide + “	7.0	0.1		0.9
	Methylguanidine sulfate + acetylhistidine	6.9	0.2		1.0
	Acetylhistidine	6.9	0.1		1.0

\* At 40° for 7 days. Experiment A, 0.75 mm of each of the nitrogenous compounds was treated with 0.5 ml. of buffer (see Tables I and II) and 2.0 ml. of 7.5 per cent formaldehyde (5 mm). Experiment B, 0.75 mm of each of the nitrogenous compounds was treated with 0.75 ml. of buffer and 6.0 ml. of 1.88 per cent formaldehyde (5 mm).

† In terms of molar equivalents of the chromogenic activity of the respective unchanged histidine derivatives.

‡ At 53° for 4 days; concentrations of reactants same as in Experiment A. Amino acids, particularly alanine, bind formaldehyde irreversibly by themselves under these conditions, so that only the colorimetric data serve as positive evidence for Mannich condensation with imidazole rings. The lack of reactivity of amide and guanidine compounds is shown by both techniques.

this purpose, since the acetyl group represented a useful “label” for analytical purposes. Additional phenolic compounds were *o*-chlorophenol, *p*-cresol, and 2,4-dimethylphenol; the fixation of the first was determined

TABLE V

*Combination of Phenols, Indoles, and Imidazoles with Proteins Containing Amino Groups by Reaction with Formaldehyde\**

Compound added	Temperature	Final pH	Apparent phenol and indole (Folin)†	Apparent phenol (Gerngross)†	Apparent histidine‡	Equivalents of added compound§
Series A. Bovine serum albumin*						
	°C.		per cent	per cent	per cent	
<i>p</i> -Cresol	23	3.5	8.7	7.4		
"	23	6.3	10.4	9.9		
"	23	7.6	10.3	9.2		
"	40	5.9	13.3	12.3		
2,4-Dimethylphenol	23	3.1	8.7	4.3		
"	23	5.9	13.5	4.4		
"	40	1.8	9.2	4.8		
"	40	3.2	12.5	3.3		
"	40	5.9	16.4	4.7		
Tyrosine	40	5.9	12.5	5.6		
Acetyltyrosine	23	3.1	9.9	5.9		1.8
"	23	5.5	12.3	6.4		3.1
"	23	7.2	13.6	6.7		4.1
<i>o</i> -Chlorophenol	23	3.3	11.6	5.6		1.9
"	23	6.1	11.9	5.5		3.2
Indole-3-acetic acid	23	3.0	9.5	4.4		
" "	23	5.4	10.4	3.9		
" "	40	5.5	12.3	3.8		
Acetyltryptophan	23	5.5	9.2	4.2		
"	23	7.2	9.3	5.1		1.4
"	40	1.8	7.0	3.8		
"	40	3.2	13.6	5.2		5.8
"	40	5.5	10.3	3.8		
"	40	7.2	8.7	3.6		2.5
Acetylhistidine	40	3.5	7.3	4.0	5.5	1.4
"	40	7.4	7.7	4.2	5.6	3.8
Histidine anhydride	40	7.5	7.6	3.1	6.3	
Controls, tyrosine, acetyltyrosine, 2,4-dimethylphenol, acetylhistidine, or acetyltryptophan, without formaldehyde	23-40	5.5-7.4	6.8	4.5	4.6	0.1
No addition + formaldehyde	23	5.8	6.0	4.3		
" " "	40	2.0-5.9	7.7	3.8	5.1	
Series B. Aminoacetyl bovine serum albumin*						
<i>p</i> -Cresol	40	6.3	6.3	4.7		
"	40	7.6	6.7	5.5		
Indole-3-acetic acid	40	5.6	7.2	4.8		

TABLE V—*Concluded*

Compound added	Temperature	Final pH	Apparent phenol and indole (Folin)†	Apparent phenol (Gerngross)†	Apparent histidine‡	Equivalents of added compound bound§
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Series B—*continued*

Acetyltryptophan	40	3.5	8.9	4.5		
	40	5.6	7.2	4.5		
	40	7.2	6.4	4.8		0.0¶
Acetylhistidine	40	7.3	6.3	4.3	4.4	1.2¶
Control, no addition + for maldehyde	40	7.5	6.0	4.2	5.0	

## Series C. Isinglass \*

	°C.		per cent	per cent	per cent
<i>p</i> -Cresol	40	5.6	3.3	2.1	
2,4-Dimethylphenol	40	5.6	5.2	0.5	
Tyrosine	40	5.6	7.2	3.3	
Indole-3-acetic acid	40	5.6	3.6	0.4	
Acetyltryptophan	40	5.6	3.8	0.6	
Controls, tyrosine or indole- 3-acetic acid, without form- aldehyde	40	5.6	1.5	0.5	
No addition + malde- hyde	40	5.6	1.7	0.3	
Unchanged			1.3	0.5	

\* For Series A and B, 1 ml. of 10 per cent protein was treated with 0.1 mM compound (the phenols in the 10 per cent solution in ethanol), 0.3 ml. of 3 M acetate or 3.4 M phosphate, and 1 ml. of 7.5 per cent formaldehyde; Series C, 2 ml. of 10 per cent protein solution and 0.2 mM addition, with 0.3 ml. of buffer and 1 ml. of 7.5 per cent formaldehyde. The amino nitrogen of bovine serum albumin is 1.18 per cent, that of the acetylated derivative (18) 0.08 per cent, and that of isinglass 0.48 per cent.

† Expressed as tyrosine. Upon analysis at two levels good agreement was obtained with the Folin method, but not with the Thomas method. The results were averaged.

‡ Histidine values include a fraction of the tyrosine present (see under "Analytical methods").

§ Per 10<sup>4</sup> gm. of protein, as determined by acetyl or chlorine analyses.

|| 120 per cent of the weight of the protein was obtained, after thorough dialysis, from the sample prepared at pH 1.8, 108 per cent from that prepared at pH 3.2, and 104 per cent from acetylated bovine serum albumin at pH 3.5. (The other preparations were obtained in 90 to 105 per cent yield.) These reaction products were colored and insoluble.

¶ These figures are, of necessity, differences of two large figures (the acetyl bovine serum albumin contains about 9 equivalents of acetyl) and thus subject to greater errors than the results obtained with unacetylated albumin.



by chlorine analyses, while increases in the Folin color characterized all products containing methylene-linked phenol or indole compounds.

The data, as summarized in Table V, show that over the pH range investigated (pH 2 to 7), the phenolic compounds were bound to the highest extent, corresponding to about half of the amino groups of serum albumin, at or near neutrality. Reactions proceeded further at 40° than at room temperature. The findings with indoles are quite similar, except that acetyltryptophan at 40° appeared to be bound maximally at pH 3. However, the insolubility and discoloration of this reaction product may suggest that complicating side reactions had occurred.<sup>6</sup> Acetylhistidine, like the phenolic compounds, was bound to the greatest extent in neutral solution. Generally, indoles and imidazoles seem to react somewhat less readily than do phenols.

The inability of aminoacetyl serum albumin to fix appreciable amounts of any of the model compounds in the presence of formaldehyde at 40° clearly establishes the crucial rôle of the protein amino groups in the cross-linking reaction. The striking difference in the chromogenic activities of the *p*-cresol and dimethylphenol derivatives of serum albumin in regard to the Folin and Gerngross reagents (Table V) represents good evidence that at least the reactions with phenols are clearly of the Mannich type.

*Introduction of Amines and Amino Acids into Gramicidin through Formaldehyde*—The participation of indole groups in condensation reactions with formaldehyde was further demonstrated by treating gramicidin, which contains almost 40 per cent tryptophan, with formaldehyde and various amines under a variety of conditions. Interaction was evident in many instances, particularly in alkaline solution, from changes in the physical properties of the reaction mixture. Altered solubilities were also noted for many products during and after their isolation. It was difficult, however, to find a reliable quantitative measure of the extent of interaction. Analyses for the polar groups introduced into the non-polar gramicidin molecule were found most useful (Table VI). Primary amines, possibly because of their tendency to cross-link through two methylene bridges, lost most of their basicity, so that basic group analyses seemed to yield only minimal values, but secondary amines remained sufficiently basic to be detected. The carboxyl groups of alanine and proline represented suitable "labels" to establish their fixation by gramicidin through formaldehyde.

The mode of linkage of these amines was not definitely established. If the reaction was of the Mannich type, the site of attachment would be the  $\alpha$ -carbon atom of the indole ring. On the other hand, formaldehyde

<sup>6</sup> Attempts to study the reaction of formaldehyde and acetyltryptophan alone in acid solutions also led to colored products difficult to characterize.

**TABLE VI**  
*Combination of Amines with Gramicidin in Presence of Formaldehyde\**

Compound added	Buffer	Final pH	Groups introduced per 10 <sup>4</sup> gm.		Apparent tryptophan†
			Acid	Basic	
Alanine	NaOH	7.8	8.5†		<i>per cent</i>  ( <i>&lt;10</i> )
	"	6.7	5.4		
	NaOAc	5.5	1.9		
	None	4.3	3.2		
	HCl	2.0	2.0		
Proline	NaOH	9.9	8.4§	0.2	(6)
	"	7.8	6.6	0.9	21
	None	4.4	2.8	1.9	
Dimethylamine	"	9.5		16.5¶	(5)
	HOAc	4.7		8.7	19
	HCl + NaOAc	3.7		4.0	
	" + HOAc	2.5		1.4	
	"	2.1		1.2	
Lysine	NaOH	8.7		3.5	15
	"	7.1		2.2	
	NaOAc	7.1		4.6	
	HCl	3.9	2.5	7.9	
	NaOAc + HCl	2.8	0.8	1.8	14
2-Aminoethylsul- furic acid	NaOH	7.5	5.8**		
Methylamine	NaOAc + HCl	3.0	0.0	2.0	(6)
	HCl	2.5		0.4	
Histidine	NaOH	6.7	1.5	0.0	
	NaOAc + HCl	2.9	1.3	1.2	
	HCl	2.8	4.4	1.7	

\* 100 mg. of gramicidin (containing 0.2 mm of tryptophan), 0.5 mm of the various additions, and 0.25 ml. of 38 per cent formaldehyde were brought into solution, if possible, in 1 to 2 ml. of 50 to 80 per cent alcohol, containing approximately 0.3 M acetate, or, at most, amounts of N NaOH or HCl equivalent to the added compound. The reaction mixtures were held at room temperature for 4 to 6 days. Heterogeneous mixtures (*i.e.* those containing lysine and alanine) were shaken at intervals. Some set to gels during the reaction period. For method of isolation see "Experimental."

† Analysis without hydrolysis, in 50 per cent acetic acid solution (0.05 per cent). Values in parentheses are based on readings of an atypical purple color. All values are approximations only ( $\pm 15$  per cent), since unhydrolyzed gramicidin and many of its derivatives give colorimetric curves which differ somewhat from those of standard tryptophan, and from one another (9).

‡ When methylol gramicidin(9) was used instead of gramicidin under identical conditions, with or without added formaldehyde, no acid groups were introduced.

§ When the product was exposed to dilute aqueous acetic acid, only 1.0 acid group was found. This material contained no unchanged tryptophan when analyzed in 50 per cent acetic acid solution (less than 10 per cent, purple). When the original product was treated with 5 N NaOH (18 hours at 100°), it contained no acid groups and about 31 per cent tryptophan.

TABLE VI—*Concluded*

|| This product after exposure to dilute acetic acid contained 1.3 acid groups and no unchanged tryptophan (less than 10 per cent, purple).

¶ Treatment with 5 N NaOH for 6 hours or 48 hours at 70°, or for 24 hours at 100°, yielded products retaining only 14.5, 12.5, or 4.2 basic groups, respectively, and 16, 13, and 31 per cent tryptophan. Dilute acetic acid gave a product containing 10.1 basic groups and less than 10 per cent tryptophan (purple).

\*\* Sulfate sulfur introduced, 6.1 equivalents.

alone in alkaline solution was found to add most probably to the imino group (9). The bulk of the evidence favors the latter group as also condensing with amines and formaldehyde in the present experiments. This is indicated by the following facts: (a) The various products retained 30 to 80 per cent of the original chromogenic activity with dimethylaminobenzaldehyde, a reaction which requires an unsubstituted 2 position in the indole nucleus.<sup>7</sup> (b) The condensation of indoles through methylene groups with amino compounds could be reversed by treatment with strong alkali, as previously observed with the N-methylol addition products.<sup>8</sup> No such lability should be expected for carbon-bound methylene groups. Thus it appears that the reaction of indoles with aminomethylols introduces methylene groups between neutral and basic nitrogen atoms. This reaction thus resembles that previously observed with amides (13), rather than the Mannich type of stable fixation of methylene groups between carbon and basic nitrogen atoms.<sup>9</sup>

*Evidence of Mannich Reaction Occurring in Proteins Alone*—Indirect evidence for the participation of phenolic and imidazole rings in the protein-formaldehyde reaction came from a study of the amount of formaldehyde bound irreversibly under various conditions. It has been well established in recent years that proteins and amino acids under progressively more rigorous reaction conditions fix progressively more formaldehyde in such a manner that it cannot be recovered by acid hydrolysis and steam distillation (31, 32). One reaction mechanism accounting for such behavior has

<sup>7</sup> N-Methylolindoles were found to give negative tests (9), but this was attributed to a possible migration of the methylol group during the test. If that interpretation is correct, then it appears that bulkier substituents do not migrate so readily from the indole nitrogen to the  $\alpha$ -carbon atom.

<sup>8</sup> The proline-formaldehyde-gramicidin condensation product appeared to be unstable even in dilute acetic acid solution (Table VI, foot-notes § and ||), but it appeared from the absence of chromogenic tryptophan that, under these circumstances, only proline was split off and methylol gramicin formed.

<sup>9</sup> A few of the gramicidin derivatives obtained in this investigation were kindly assayed for bacteriostatic activity (against *Staphylococcus aureus*) by J. C. Lewis of this Laboratory. Their activities ranged from 13 to 88 per cent of that of the original gramicidin, but they were also similarly toxic to mice.

recently been elucidated as the addition of formaldehyde to protein indole (9) and phenol<sup>4</sup> groups in alkaline solution. These reactions, however, do not occur at neutrality. Acylhistidines by themselves do not bind formaldehyde at any pH studied (24, 23). Thus presumably the imidazole groups of proteins are by themselves unreactive.

When the amounts of formaldehyde fixed irreversibly by a number of

TABLE VII

*Comparison of Amounts of Formaldehyde Bound Irreversibly by Various Proteins at 40° and pH 7 with Their Contents of Relevant Amino Acids\**

	Equivalents per 10 <sup>4</sup> gm.			
	Formaldehyde bound	Contents of		
		Histidine	Tyrosine	NH <sub>2</sub> -N
Insulin.....	5.3	3.4	6.8	5.0
Globin.....	4.1	5.2	1.9	7.5
Protamine†.....	4.0	0.0	0.0	0.0
Serum albumin (bovine).....	3.3	2.5	3.1	8.9
Silk sericin.....	2.7	1.2	3.0	2.2
Feathers (chicken, ground).....	2.3	0.3	1.9	1.5
Hoof (bovine, powdered).....	2.2	1.2	2.9	4.5
Wool (ground).....	1.6	0.4	2.7	1.8
Silk fibroin (ground).....	1.3	0.2	6.6	0.7
Gelatin.....	1.0	0.4	0.7	4.0
N-Acetyl globin.....	1.0	5.2	1.9	2.0
“ serum albumin.....	0.7	2.5	3.1	0.5
Barium sulfate.....	0.2	0.0	0.0	0.0
Nylon.....	0.0	0.0	0.0	0.1

\* 100 mg. of protein were treated with 1.0 ml. of a reaction mixture containing 2 ml. of 7.5 per cent formaldehyde, 3 ml. of M phosphate, pH 7.0, and 23 ml. of water in sealed test-tubes for 1 month at 40°. The total sample was then subjected to analysis by distillation from sulfuric acid solution (1, 20). The proteins selected were all low in tryptophan content (<0.7 equivalent per 10<sup>4</sup> gm.), so that the rôle of the indole groups (9) in this reaction could be disregarded.

† See foot-note 10 in the text.

proteins of greatly varying composition were compared after reaction at pH 7 (1 month at 40°), those proteins which were richest in histidine and tyrosine, and also contained many amino groups, bound the most formaldehyde, though not appreciably more than was equivalent to their content of such groups (Table VII).<sup>10</sup> The low amounts of formaldehyde fixed by

<sup>10</sup> The large amount of formaldehyde irreversibly bound by salmine under these conditions represents an exception. The mechanism of this reaction is not understood.

silk fibroin and by aminoacetyl serum albumin or globin, all of which contain appreciable amounts of tyrosine or histidine but little amino nitrogen, illustrate that formaldehyde is fixed by the cyclic residues through the Mannich reaction, rather than by simple addition to the ring.

The authors acknowledge the untiring technical assistance of E. D. Ducay.

#### SUMMARY

In neutral or slightly acid solution, formaldehyde introduces methylene bridges between amines on the one hand and the reactive CH groups of phenolic and imidazole rings on the other. The phenols react to completion within a few days at room temperature. The linkage is resistant to acid hydrolysis.

Experiments with gramicidin suggest that condensations joining indoles, amines, and formaldehyde under similar conditions may occur with the NH group of the indole ring.

These reactions take place with similar ease also in proteins containing the necessary groups. They supply a means of introducing a great variety of reactive cyclic compounds stably into proteins, and of amino compounds into gramicidin.

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# A POLARISCOPIC STUDY OF GLUTAMIC ACID AND PYRROLIDONECARBOXYLIC ACID\*

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Wilson and Cannan studied the interconversion of glutamic acid and pyrrolidonecarboxylic acid in detail by means of changes in amino nitrogen (1), and the system so characterized was utilized by Olcott for the development of a method for the determination of glutamic acid (2). In neither case, however, were temperatures above 125° studied. We have found that glutamic acid can be converted much more rapidly at 142°. Since such temperatures are known to produce profound changes in many substances, an investigation of the optical rotation of this system was undertaken.

## EXPERIMENTAL

Weighed amounts of L-glutamic acid, recrystallized at pH 3.2, were placed in pressure flasks and water was added to the desired concentrations. The flasks were immersed in an oil bath with thermostatic control and were removed momentarily and shaken after 5 minutes. Heating was then continued for specified times. After being removed from the bath and cooled, the pH of the solutions was determined with a glass electrode, and they were then transferred to volumetric flasks and diluted to volume. Samples of the diluted solutions were taken for duplicate determinations of amino nitrogen in a Van Slyke volumetric apparatus.

Observations were made at four temperatures between 127–142° in solutions containing 2 to 20 per cent glutamic acid. Since concentration did not affect the rate significantly, the results of all of the experiments at each temperature were combined. The average values appear in Table I, where the data of Olcott (2) for 0.2 per cent glutamic acid at 125° are included for comparison. Maximum dehydration of glutamic acid is achieved in 2 hours at 139° and 142°. In fact, at the latter temperature it has been found that the conversion to pyrrolidonecarboxylic acid is completed in 75 to 90 minutes. Although with isoelectric glutamic acid

\* This work was started at the University of Toledo laboratory of the research department of the International Minerals and Chemical Corporation. We are indebted to Dr. P. D. V. Manning, vice president and research director, International Minerals and Chemical Corporation, Chicago, for permission to use some of the results presented here.



at pH 3.2 only 92 per cent is converted at equilibrium, 96 per cent of the glutamic acid can be converted in solution at an initial pH of 4 without affecting the rate significantly.

In order to calculate the rotation of solutions containing both glutamic acid and pyrrolidonecarboxylic acid, the specific rotation of both of these substances was determined. In Fig. 1, Curve A shows the value of  $[\alpha]_D^{25}$  in solutions of L-pyrrolidonecarboxylic acid between pH 2 and 11, and Curve B the values in solutions containing 2 to 12 per cent hydrochloric acid. The specific rotation of L-glutamic acid had a value of  $+31.4^\circ$  in solutions containing 5 to 12 per cent hydrochloric acid, and the value was at a minimum close to  $-5.0^\circ$  at pH 7.

TABLE I

*Conversion of Isoelectric Glutamic Acid to Pyrrolidonecarboxylic Acid at Different Temperatures*

Time hrs.	Conversion				
	125°	127°	135°	139°	142°
	per cent	per cent	per cent	per cent	per cent
1	55	65.9	76.2	76.0	85.5
2	80	81.6	90.0	91.4	92.5
3	88	89.0	91.7	91.8	92.5
4	92	92.2	91.8	91.9	92.7
5			91.9		
6		92.8		91.9	92.7
8		92.9		91.9	93.0

From Olcott (2).

The rate of racemization of glutamic acid on being boiled in solutions in which partial dehydration to pyrrolidonecarboxylic acid may occur was studied in the following way. Solutions containing 10 per cent glutamic acid were boiled under a reflux at an initial pH of 1, 2, 7, and 12, and samples were removed periodically for the determination of the pH and amino nitrogen. The balance of the samples was then adjusted with hydrochloric acid and the rotation was determined in 4 dm. tubes in either 5 or 10 per cent acid. Some of the results are presented in Table II. It is seen that the values of rotation found in solutions boiled many hours at low pH agree quite well with those calculated on the assumption that racemization is not significant. Furthermore, the absence of significant racemization in the solutions boiled at pH 7 was confirmed by the value of  $+29.8^\circ$  for the specific rotation of the glutamic acid after regeneration in boiling hydrochloric acid. However, it is evident from the poor agreement of the values in solutions boiled at pH 12 that racemization takes place rapidly under these conditions.

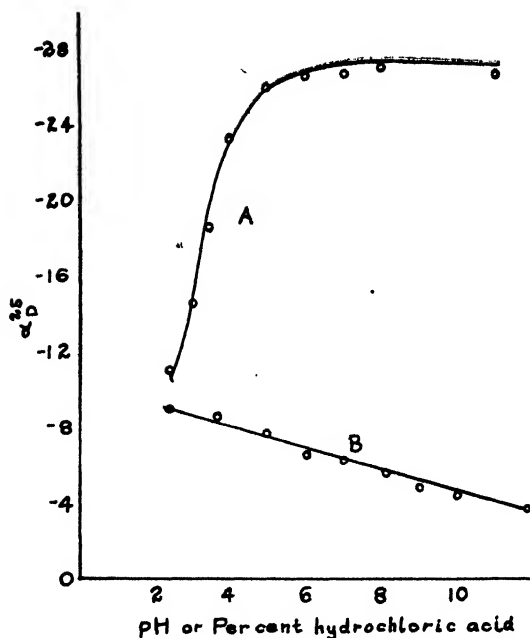


FIG. 1. The rotation of pyrrolidonecarboxylic acid. Curve A, abscissa represents pH; Curve B, abscissa represents per cent hydrochloric acid.

TABLE II

*Effect on Glutamic Acid of Being Boiled in Acid and Alkaline Solutions*

Time	pH	Glutamic acid	Rotation, $\alpha^\circ$	
			Found	Calculated
<i>hrs.</i>		<i>gm. per 100 ml.</i>	<i>degrees</i>	<i>degrees</i>
0	1.17	5.00	+18.2	+18.1
3	0.80	3.65	+12.0	+12.2
43	0.74	3.57	+11.4	+11.8
91	0.69	3.62	+11.5	+12.0
0	2.17	5.00	+18.1	+18.1
6	1.43	2.56	+7.4	+7.5
31	1.27	2.12	+5.4	+5.6
101	1.20	2.24	+5.5	+6.0
0	7.10	5.00	+18.1	+18.1
170	7.42	3.22	+9.6	+10.3
0	11.5	5.00	+18.1	+18.1
6	12.4	4.53	+15.9	+16.2
29	12.1	4.48	+14.8	+16.0
60	12.0	4.41	+13.5	+15.7
110	12.0	4.02	+10.7	+14.1

\* Ventzke scale.

The resistance of glutamic acid to racemization at 127° and 142° was studied in a similar manner by heating isoelectric glutamic acid in 20 per cent solution. After the pH was determined, the solutions were diluted with water for the estimation of amino nitrogen in the Van Slyke apparatus. Hydrochloric acid was then added for the determination of rotation in 10 per cent acid. In addition, the acidified solutions were boiled and the amino nitrogen contents and rotation were again determined after regeneration. Table III shows good agreement between the experimental and calculated values of rotation, but the values of the specific rotation of the regenerated glutamic acid are somewhat low.

Since the equilibrium in the region of pH 7 allows nearly complete conversion to pyrrolidonecarboxylic acid (1), the rates of dehydration and of

TABLE III  
*Effect of Heating Solutions of Isoelectric Glutamic Acid at 127° and 142°*

Time	Temperature	pH	Rotation, $\alpha^*$		Regenerated glutamic acid, $[\alpha]_D^{25}$
			Found	Calculated	
<i>hrs.</i>	<i>°C.</i>		<i>degrees</i>	<i>degrees</i>	<i>degrees</i>
1.3	127	2.48	+4.2	+4.4	+29.6
3.3	127	2.07	-0.5	-0.4	+29.1
5.3	127	2.07	-0.6	-0.5	+28.4
7.3	127	2.07	-0.5	-0.5	+28.4
1	142	2.01	-0.5	-0.7	+29.5
2	142	2.01	-0.7	-0.9	+28.1
3	142	2.01	-0.7	-0.9	+27.4
4	142	2.01	-0.6	-0.9	+27.5

\* Ventske scale.

racemization of glutamic acid were studied in neutral solutions at 142°. The solutions were heated for various times up to 72 hours, after which the amino nitrogen was determined and the rotation was measured both at pH 7 and in 10 per cent hydrochloric acid. At least 30 hours are required for the completion of the reaction even at 142°, and the prolonged heating at this temperature seems to cause some racemization or destruction in the system. At 17 hours, when the conversion was 80 per cent, a comparison between the experimental and calculated values of rotation indicated that racemization was not significant. However, the agreement was not good at 54 hours, and the specific rotation was only +26.0° after the glutamic acid was regenerated.

Observations were also made on the hydrolysis of pyrrolidonecarboxylic acid at room temperature. Solutions of 5 per cent pyrrolidonecarboxylic acid in 6 and 12 per cent hydrochloric acid were allowed to stand for 56

days at 25–30°. The rotation was determined periodically in the acid solutions and amino nitrogen was determined after neutralization. The degree of conversion was then calculated from both sets of data. There was good agreement for 10 days, during which about 60 per cent of the pyrrolidonecarboxylic acid was hydrolyzed. However, the results became increasingly less consistent, and after 46 days only 85 to 90 per cent was shown to be hydrolyzed from the values of rotation, although the process was complete according to the values for amino nitrogen.

#### SUMMARY

1. Data are presented to show that the dehydration of L-glutamic acid to L-pyrrolidonecarboxylic acid is accomplished in 2 hours at 142° with little alteration of the optical rotation of the system.

2. The specific rotation of L-pyrrolidonecarboxylic acid has been determined from pH 2 to 11 and in solutions containing 2 to 12 per cent hydrochloric acid.

3. The effect of boiling at acid and alkaline reactions on the optical rotation of the system glutamic acid-pyrrolidonecarboxylic acid has been studied by a comparison of the observed rotation with the values calculated from the composition of the solutions. At pH 1, 2, and 7 the agreement is good for many hours and indicates that racemization is not significant, but the results show that racemization takes place rapidly at pH 12.

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# ON THE PROTEOLYTIC ENZYMES OF ANIMAL TISSUES

## VIII. AN INTRACELLULAR ENZYME RELATED TO CHYMOTRYPSIN\*

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In previous papers of this series (1, 2), evidence was offered for the presence of two types of endopeptidases in extracts of animal tissues such as spleen or kidney. One group of enzymes exhibits specificity requirements closely related to those of crystalline pepsin (3), and these enzymes have therefore been designated "pepsinases" (*cf.* also (4)). Since the protein-splitting enzymes of animal tissues are also termed cathepsins, the pepsinases derived from beef spleen and from swine kidney were called beef spleen cathepsin I and swine kidney cathepsin I respectively. The other group of tissue endopeptidases is characterized by a specificity similar to that of crystalline pancreatic trypsin, which readily hydrolyzes substrates such as benzoyl-L-argininamide (BAA). Intracellular proteinases of this type were assigned the name cathepsin II. As noted previously, cathepsins I and II differ with respect to their response to activators such as cysteine (1).

It has long been clear (5) that, in addition to the two endopeptidases cited above, tissue extracts contain other members of this group of enzymes which await characterization with respect to their specificity of action. For example, the enzymes responsible for the hydrolysis of substrates such as benzoylglycinamide, carbobenzoxy-L-isoglutamine (1), carbobenzoxy-L-serinamide (6), etc., by crude extracts of animal tissues have yet to be studied carefully.

In the present communication, data are presented to show that swine kidney extracts contain an enzyme closely related in specificity to crystalline pancreatic chymotrypsin. It has been reported previously that crystalline chymotrypsin hydrolyzes glycyl-L-phenylalaninamide (GPA) and glycyl-L-tyrosinamide (GTA) at the CO-NH linkage involving the carbonyl group of the aromatic amino acid (7), and that carbobenzoxy-L-tyrosylglycinamide is split to yield carbobenzoxy-L-tyrosine and glycine (8). In addition to this endopeptidase activity, crystalline chymotrypsin also exhibits aminopeptidase activity toward substrates such as

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† Alexander Brown Coxe Memorial Fellow, Yale University, 1946-48.

L-phenylalaninamide (PA), L-tyrosinamide (TA), L-phenylalanylglycinamide (PGA), and L-tyrosylglycinamide (TGA) (7).

For the present studies, two enzyme preparations from swine kidney were employed. One of these was obtained by the procedure described previously (1, 2) and will be referred to as enzyme Preparation A. The other enzyme preparation (Preparation B) was obtained by the addition of yeast nucleic acid to a dialyzed solution of Preparation A, followed by elution of the resulting precipitate with a solution of benzoyl-L-argininamide

TABLE I

*Action of Swine Kidney Enzyme Preparations on Synthetic Substrates*

Cysteine (0.004 M) was present in all cases, except where indicated otherwise.

Substrate	pH	Proteolytic coefficient $\times 10^{10}$ *		
		Crude extract†	Preparation A	Preparation B
Benzoyl-L-argininamide .....	5	1.5	3.2	8.9
Glycyl-L-phenylalaninamide .....	5		4.3	66
			2.5‡	
Carbobenzoxy-L-glutamyl-L-tyrosine .....	5	0.5‡	0‡	0‡
Carbobenzoxy-L-isoglutamine .....	5	0.5	0	0
Benzoylglycinamide .....	5	0.33	0	0
Carbobenzoxyglycyl-L-phenylalanine .....	5	0.7	2.0	0
L-Leucinamide .....	5	2.3	3.3	0
	8	86§	2.6§	0§
Diglycylglycine .....	5	67.5	5.5	0.4
	8	22‡	1.7‡	0‡

\* The term "proteolytic coefficient," as used in this table, has been defined previously (10) as the first order rate constant ( $K^1$ ) per mg. of protein-N per cc. of test solution.

† This represents a crude aqueous extract of fresh swine kidney, prepared as previously described (1, 2).

‡ No cysteine added.

§ 0.001 M  $\text{MnSO}_4$  present; no cysteine added.

in 0.06 M citrate buffer (pH 4.3). The arginine derivative may be assumed to act in the elution procedure in a manner analogous to that of protamine, which has been used previously for the decomposition of protein nucleates (9). In the case of enzyme Preparation B, the eluted proteins are fractionated with ammonium sulfate, the fraction precipitated between 45 and 75 per cent of saturation with ammonium sulfate being collected and dialyzed.

As will be seen from Table I, the partial purification leading to Preparation B has largely eliminated the enzyme activity toward carbobenzoxy-L-glutamyl-L-tyrosine (cathepsin I), carbobenzoxyglycyl-L-phenylalanine

(carboxypeptidase), L-leucinamide (aminopeptidase), and diglycylglycine (tripeptidase (11)), as well as toward carbobenzoxy-L-isoglutamine and benzoylglycinamide. On the other hand, the cathepsin II activity toward BAA has been increased in the course of the procedure, as has the activity toward GPA.

It may be added that, in the presence of cysteine, the hydrolysis of GPA follows the kinetics of a first order reaction and that the proteolytic co-

TABLE II

*Action of Swine Kidney Enzyme Preparation B on Substrates of Chymotrypsin pH 5 (citrate buffer).*

Substrate	Enzyme concentration*	Cysteine	Time	Hydrolysis	
				Titration	Ninhydrin
	mg.	M	hrs.	per cent	per cent
Glycyl-L-phenylalaninamide . . . . .	0.074	0.004	0.6	32	1
			2	79	4
			24	111	17
		None	1.9	29	4
			5.5	49	17
			25.8	103	44
Glycyl-L-tyrosinamide . . . . .	0.074	0.004	1.5	74	
			26.5	99	29
L-Phenylalaninamide . . . . .	0.124	0.004	2.5	33	
			6.3	63	
			2.5	12	
			6.3	22	
L-Tyrosinamide . . . . .	0.124	0.004	2.1		11
			6.3		36†
		None	2.1		7
			6.3		10
L-Phenylalanylglycinamide . . . . .	0.074	0.004	4.7	36	6
			24.2	63	18
L-Tyrosylglycinamide . . . . .	0.152	0.004	6.2	25†	

\* Expressed as protein N per cc. of test solution.

† Crystals of tyrosine separated after 20 hours.

efficient (10) for the hydrolysis of this substrate by Preparation B is about 30 times as great as the value previously found for the hydrolysis of GPA by crystalline chymotrypsin.

Further studies on the proteolytic activity of enzyme Preparation B showed that, in the presence of cysteine, there was appreciable hydrolysis, not only of GPA, but also of other substances previously recognized to be substrates for crystalline chymotrypsin. In Table II there are presented data on the cleavage of GTA, PA, TA, PGA, and TGA. In order to de-



termine the site of hydrolysis of the dipeptide amides, the rate of hydrolysis was followed by titration of the liberated carboxyl groups (12) as well as by the determination of free amino acids (13). As will be noted from Table II, the hydrolysis of GPA and of GTA, in the presence of cysteine, largely involves the scission of the bond involving the carbonyl group of the aromatic amino acid. On prolonged incubation, however, there is observed some cleavage to yield free amino acids. A clue to the nature of the enzyme responsible for the latter effect is provided by experiments in which GPA was subjected to the action of Preparation B in the absence of added cysteine. Under these circumstances, the rate of formation of free amino acids is notably more rapid. This suggests that, in addition to a cysteine-activatable endopeptidase similar in specificity to chymotrypsin, Preparation B also contains an aminopeptidase whose action is inhibited by cysteine. Recent studies have shown that several aminopeptidases are activated by metal ions and are inhibited by substances, such as cysteine, which form complexes with metals (14). Further work is required before the relationship of the aminopeptidase acting on GPA to known aminopeptidases can be adequately assessed. It may be added that the dipeptide glycyl-L-phenylalanine is hydrolyzed by Preparation A, and that this action is strongly inhibited by cysteine.

In addition to its endopeptidase action on GPA, Preparation B hydrolyzes carbobenzoxy-L-tyrosylglycinamide and carbobenzoxyglycyl-L-tyrosylglycinamide. Carbobenzoxy-L-tyrosine was isolated from the enzymatic hydrolysate of the former compound by the procedure described in (8).

As pointed out previously, chymotrypsin exhibits aminopeptidase activity toward PA and TA. A similar action is shown by enzyme Preparation B, and it is of interest that cysteine is an activator for these hydrolyses (Table II). This behavior differentiates the aminopeptidase action on GPA, which is inhibited by cysteine, from the splitting involving aminopeptidase action at the carbonyl group of an aromatic amino acid.

In Table II, there are also presented data on the hydrolysis of PGA and TGA by enzyme Preparation B. It will be seen that, in the case of PGA, extensive hydrolysis occurs at the CO-NH bond involving the carbonyl group of the glycine residue. A similar result was noted for crystalline chymotrypsin (7), which showed slight endopeptidase action on PGA. In the case of the swine kidney preparation, however, the endopeptidase activity appears to be considerably greater than the aminopeptidase action.

Attempts to separate the endopeptidase and aminopeptidase activities of crystalline chymotrypsin were not successful (7), and the possibility was raised that this enzyme is a protein that exhibits two separate enzymatic specificities. On the other hand, in the case of Preparation B, partial inactivation of the aminopeptidase activity toward PA could be effected with-

out any concomitant decrease in the endopeptidase activity toward GPA (Table III). Furthermore, comparison of the relative rates of hydrolysis of GPA and PA by enzyme Preparations A and B supports the conclusion that these two substrates are split by different enzymes of swine kidney. It may be added that, as in the case of GPA, the hydrolysis of PA by these enzyme preparations follows the kinetics of a first order reaction.

The data in Table III clearly differentiate cathepsin II from the swine kidney enzymes that act on GPA and PA. It will be noted that, after exposure of Preparation B to 52° for 30 minutes, its activity toward BAA decreased by 58 per cent, while the activity toward GPA remained unchanged. As a result of this treatment, the action on PA was decreased by 26 per cent. Experiments are in progress to determine whether the

TABLE III

*Partial Inactivation of Swine Kidney Enzyme Preparation B*

A dialyzed solution of enzyme Preparation B was warmed to 62° for 20 minutes and then chilled in ice water. The resulting enzyme solution is referred to as Preparation B<sub>1</sub>. Another aliquot of Preparation B was warmed to 52° for 30 minutes and then chilled. This enzyme solution is termed Preparation B<sub>2</sub>. The hydrolytic experiments were conducted at pH 5 (citrate buffer) and in the presence of cysteine (0.004 M).

Substrate	Proteolytic coefficient		
	Preparation B	Preparation B <sub>1</sub>	Preparation B <sub>2</sub>
Glycyl-L-phenylalaninamide.....	0.071	0.072	0.068
L-Phenylalaninamide.....	0.0068	0.0046	0.0050
Benzoyl-L-argininamide.....	0.0078		0.0033

relative heat stability of the chymotrypsin-like endopeptidase of swine kidney extracts can be used in the further purification of this enzyme.

*Action of Enzyme Preparation B on Proteins*—Advantage has been taken of the availability of the partially purified cathepsin Preparation B to test its action on proteins. It was of interest to find that no appreciable cleavage of the peptide bonds of gelatin or of  $\beta$ -lactoglobulin occurred in the absence of added cysteine. As will be noted from Table IV, these proteins were readily split in the presence of cysteine. When Preparation B was warmed to 52° for 30 minutes, the rate of protein hydrolysis, as measured by the liberation of amino nitrogen, is markedly decreased. The data in Table IV show that the extent of inactivation of the enzymes acting on gelatin and  $\beta$ -lactoglobulin is similar to the extent of inactivation of cathepsin II. Since the chymotrypsin-like endopeptidase of swine kidney is active in the absence of added cysteine and is stable at 52°, it may be surmised

that this enzyme plays a secondary rôle in the hydrolysis of the two proteins under study. The data in Table IV support the view, therefore, that

TABLE IV

*Action of Swine Kidney Enzyme Preparation B on Proteins*

Concentration of protein substrates, 0.49 mg. of protein N per cc. of test solution; pH 5 (citrate buffer); cysteine (0.004 M).

Enzyme, protein N per cc. test solution	Increase in NH <sub>2</sub> -N in 3 hrs.*				Inacti- vation	K <sup>1</sup> <sub>BAA</sub> × 10 <sup>3</sup>		Inacti- vation
	Control		Inactivated†			Control	Inacti- vated†	
	Gelatin	β-Lacto- globulin	Gelatin	β-Lacto- globulin				
	mg.	mg.	mg.	mg.				
0.128	0.089		0.056		37	1.00	0.60	40
0.198		0.043		0.022	49	1.85	0.80	58

\* The hydrolysis of the proteins was followed by means of the Van Slyke nitrous acid method. The results are expressed in terms of mg. of amino nitrogen per cc. of test solution.

† The enzyme solution used as the control was heated to 52° for 30 minutes, as described in Table III.

TABLE V

*Effect of Crystalline Trypsin Inhibitor on Hydrolysis of Benzoyl-L-argininamide by Trypsin and Cathepsin II*

Enzyme	Inhibitor* con- centration per cc. test solution	$K^1_{\text{BAA}} \times 10^3$	Inhibition
	mg.		per cent
Crystalline trypsin (0.026 mg. pro- tein N per cc. test solution)†	0	2.6	
	0.0226	1.8	31
	0.0456	1.3	50
Swine kidney Preparation A (1.46 mg. protein, N per cc. test so- lution)‡	0	2.5	
	0.0226	2.6	0
	0.0456	2.6	0

\* The authors are indebted to Dr. M. Kunitz for a 3 times crystallized sample of this material.

† Tested at pH 7.5 (phosphate buffer).

‡ Tested at pH 5 (citrate buffer) and in the presence of cysteine (0.004 M).

cathepsin II is able to hydrolyze the peptide bonds of proteins such as gelatin and  $\beta$ -lactoglobulin.

*Effect of Crystalline Soy Bean Inhibitor on Trypsin and on Cathepsin II*—Kunitz (15) has isolated from soy beans a crystalline protein which inhibits the action of pancreatic trypsin on proteins. The data in Table V show

that this inhibitor also decreases the rate of action of trypsin on BAA. At comparable levels of inhibitor concentration, however, no diminution of cathepsin II activity on BAA can be observed. This difference in the behavior of the two homospecific enzymes (trypsin and cathepsin II) may be added, therefore, to the previously recognized differences in their pH optima and response to activators such as cysteine (2).

#### EXPERIMENTAL

*Swine Kidney Enzyme Preparation B*—5 gm. of Preparation A (1, 2) were dissolved in 25 cc. of distilled water and dialyzed against distilled water for 12 hours. The precipitate which appeared was removed by centrifugation and the supernatant was diluted to 50 cc. To 45 cc. of this solution there was added an aqueous solution of yeast nucleic acid (550 mg. in 22 cc.; pH 6.5). 0.5 M acetic acid was then added with mechanical stirring until the pH was 4.2 to 4.3. After the mixture had been allowed to stand at 5° for 12 hours, the precipitate which had separated was filtered with the aid of 200 mg. of Filter-Cel, and stirred with 30 cc. of ice-cold water. The suspension was centrifuged at once and to the precipitate there were added an aqueous solution of BAA (20.8 mg. in 0.5 cc.) and 10 cc. of 0.06 M citrate buffer (pH 4.3). The mixture was stirred at 42° for 10 minutes, centrifuged, and the sediment reextracted with fresh solutions of BAA and of citrate buffer. The eluates from both extractions were combined (volume, 21 cc.) and there were added, with stirring, 5.8 gm. of ammonium sulfate. The resulting precipitate was centrifuged off, and to 20 cc. of the supernatant there were added, with stirring, 25 cc. of saturated ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 10 cc. of ice-cold water. The enzyme solution was then dialyzed against 1 per cent sodium chloride for 18 hours.

*Enzymatic Studies*—The methods employed in following the rate of hydrolysis of the synthetic substrates were the same as those described in (7), except that the temperature was 38° in all cases. The synthesis of the peptides and peptide derivatives used in the present studies has been described in previous papers of this series.

#### SUMMARY

There has been obtained, from aqueous swine kidney extracts, a partially purified enzyme preparation which hydrolyzes synthetic peptide derivatives previously found to be substrates for crystalline chymotrypsin. Evidence has been offered for the presence, in such purified preparations, of an endopeptidase and an aminopeptidase which, in the presence of cysteine, hydrolyze peptide bonds involving the carbonyl group of tyrosine or phenylalanine. This chymotrypsin-like action may be differentiated from the

trypsin-like activity of cathepsin II, also present in purified swine kidney preparations, by a marked difference in their stability to heat.

The partially purified swine kidney preparation hydrolyzes peptide bonds of proteins (gelatin,  $\beta$ -lactoglobulin) and the available evidence suggests that cathepsin II may be involved in this action.

The crystalline trypsin inhibitor from soy beans has no effect on the action of cathepsin II on benzoyl-L-argininamide, although it inhibits the hydrolysis of this substrate by crystalline trypsin.

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# THE ARGINASE ACTIVITY OF ISOLATED CELL NUCLEI

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Arginase appears to be one of the first enzymes which was studied in cell nuclei isolated on a relatively large scale by physicochemical methods. This enzyme was measured in isolated nuclei of liver cells by Behrens (1) who reported that the arginase activity per dry weight of isolated nuclei was about equal to the arginase activity of cytoplasm per dry weight in the case of mammalian liver. It was reported later from this laboratory (2) that the activity of arginase per dry weight of nuclei isolated by a different method from rat liver was 40 to 50 per cent of its activity per dry weight of whole rat liver. Recently the method of isolating nuclei of rat liver which was previously used in this laboratory has been improved, chiefly by better control of the pH and by employing a low temperature throughout the isolation procedure<sup>1</sup> (3). It was discovered that the arginase activity of a sample of nuclei isolated from normal rat liver by the improved method was considerably higher than previously reported, and therefore it appeared desirable to reinvestigate the question of the activity of this enzyme in isolated liver cell nuclei. Moreover, it has recently been possible to extend the improved method of isolating liver cell nuclei to kidney and pancreas, and therefore it has been possible to extend the work on arginase to nuclei isolated from these two tissues. The chief results of the new investigation are (a) that the activity of arginase per dry weight of nuclei isolated from rat liver cells is slightly higher than the corresponding activity per dry weight of whole rat liver; (b) that the arginase activity of nuclei isolated from dog, lamb, rat, or chicken kidney is negligible, although arginase activity can be measured in the whole kidney of all of these species; and (c) that the arginase activity of whole chicken liver and whole sheep pancreas, as well as of nuclei isolated from these tissues, is extremely low or zero. Experiments also have been carried out to determine whether the arginase of isolated liver cell nuclei is an integral part of the nucleus, or whether it is

<sup>1</sup> The method described in this paper for isolating cell nuclei from dog or lamb kidney is essentially the same as the improved method for isolating cell nuclei from rat liver (3). A few layers of fine cheese-cloth can be substituted for the flannel used in straining the kidney preparation, and the material need be passed through this cheese-cloth only once. Also, one sedimentation is sufficient for removing whole cells and fiber from the liver cell nuclei.

merely acquired by adsorption or chemical combination from the cytoplasmic arginase during the process of isolating the nuclei. It has been concluded from this part of the work that in all probability the arginase is an integral part of the nucleus.

#### EXPERIMENTAL

*Preparation of Isolated Nuclei of Rat Liver Cells*—The method described elsewhere<sup>1</sup> (3) was employed.

*Preparation of Isolated Nuclei of Kidney (Dog, Lamb, Rat, and Chicken)*—Most of the medulla of the kidneys is removed and as much fibrous tissue as possible is removed and discarded. The kidneys are then cleaned of debris and are frozen in the freezing compartment of an electric refrigerator. 50 gm. of frozen cleaned kidney are cut into small pieces and blended in the Waring blender<sup>2</sup> for 10 seconds with 100 ml. of ice-cold distilled water. 4.2 ml. of 0.1 M citric acid are added during the next 10 seconds of blending. The suspension is filtered immediately through one layer of fine cheese-cloth; the residue on the cloth is washed with 25 ml. of ice-cold distilled water. The filtrate is then refiltered through one layer of fine flannel. During the filtration the suspension must be kept cold either by using an ice bath or by working in a cold room. The filtering mixture may be agitated with a stirring rod but the residue should not be squeezed out at any time. The total time of filtration should not exceed 15 minutes. The filtered material is blended for 5 minutes. During this time 125 gm. of ice are added. The final temperature should not exceed 5° and the pH should be between 5.8 and 6.0. Then the suspension is filtered through two layers of fine cheese-cloth and placed in two 250 ml. centrifuge tubes and centrifuged at approximately 2000 R.P.M. for 20 minutes. The supernatant is decanted up to the loosely packed sediment and discarded. Now the sediments of the two cups are combined and thoroughly stirred with enough ice-cold distilled water to make a total volume of approximately 200 ml. The suspension is then centrifuged in one cup for 15 minutes at approximately 1700 R.P.M. The supernatant is decanted from the packed material and discarded. The washings with 200 ml. of ice-cold distilled water are repeated twice and the suspensions are centrifuged at approximately 1400 and 1000 R.P.M. for 10 and 7 minutes respectively. After the last washing, the sediment consists of only nuclei, fiber, and some very fine particles. The suspension is made up to 100 ml. with ice water and sedimented in a sediment-

<sup>2</sup> The speed of the Waring blender is fairly critical. The old style blender which draws 2 amperes of current operates at the correct speed, but the new style blender which draws 3 amperes must be operated with a rheostat in series which reduces the operating voltage to about 95 volts, in order to avoid disintegrating the nuclei themselves.

ing apparatus (Fig. 1) for 2 hours in the cold room. At the end of this time the top 95 ml. are separated from the 5 ml. at the bottom and centrifuged for 5 minutes at approximately 1000 R.P.M. The nuclei are suspended in a small amount of ice-cold water and examined under the microscope.

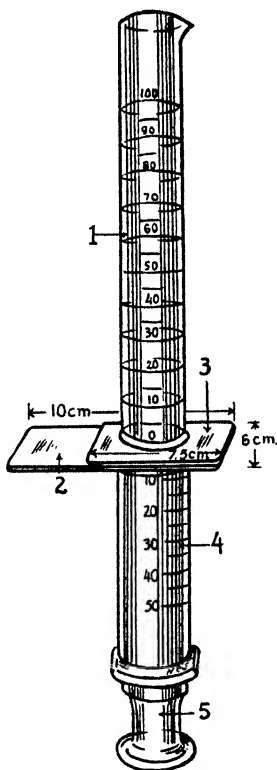


FIG. 1. Sedimentation apparatus. 1, 100 ml. graduated cylinder with bottom removed, cemented to a ground glass plate; 2, lower ground glass plate with circular hole of the same diameter as the hole in the upper plate, cemented to a graduated 50 ml. syringe; 3, upper ground glass plate with a circular hole of the diameter of the graduated cylinder, displaceable laterally in order to imprison the sedimented material; 4, 50 ml. graduated syringe with bottom removed, cemented to the lower ground glass plate and to be mounted in some suitable supporting frame; 5, plunger of the syringe for adjusting the volume of heavy sediment to be removed.

Should the preparation still be contaminated with fiber, the sedimentation procedure must be repeated, this time with only 50 ml. of suspension, allowing the material to undergo sedimentation for only 1 hour (in the cold room). If any fine particles remain after sedimentation, they are removed by centrifuging with 200 ml. of ice-cold water at 700 to 1000 R.P.M. This pro-



cedure unfortunately may also cause some loss of smaller nuclei. The final sediment, consisting of isolated nuclei, should be suspended in a small amount of ice-cold redistilled water and placed in the refrigerator.

A photograph of dog kidney cell nuclei prepared as described above is shown in Fig. 2.

*Preparation of Isolated Nuclei from Chicken Liver*—These were prepared with the use of the Waring blender in the same manner as for the preparation of other liver nuclei. The nuclei were very free from cytoplasm and quite small in size.



FIG. 2. Photograph of dog kidney cell nuclei

*Preparation of Isolated Cell Nuclei from Sheep Pancreas*—Essentially the same method was used as is described above for the preparation of nuclei from dog and sheep kidney.

*Method of Determining Arginase*—The procedure previously employed in this laboratory (2) was used, with slight modification of washing the dioxanthyl urea only once with 50 per cent acetic acid and once with water, instead of twice with 50 per cent acetic acid and twice with water. Also, in most cases a substrate concentration of 3 per cent arginine carbonate was used instead of 1 per cent arginine carbonate. The relationship between the mg. of dioxanthyl urea produced by a given amount of liver homogenate or of nuclei with 3 per cent substrate and that produced by using 1 per cent substrate is essentially linear, as is shown in Fig. 3. All weights of dioxanthyl urea obtained with 3 per cent substrate were converted by the curve in

Fig. 3 to weights of dioxanthyl urea that would have been obtained with 1 per cent substrate, and the values of  $K$  per gm. for arginase which appear in Tables I, II, and IV were calculated on the basis of 1 per cent substrate, in order to obtain results which are comparable to those previously published (2). Although the use of 3 per cent arginine carbonate gives somewhat greater amounts of dioxanthyl urea than does 1 per cent arginine carbonate, we do not think the waste of substrate is justified and recommend the use of 1 per cent substrate for this reason. In order to change the kinetics of the reaction from first order to zero order, which is always a desirable procedure in enzyme work, it would be necessary to employ substrate concentrations of 5 per cent or higher.

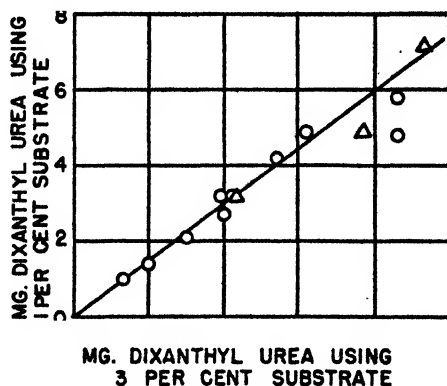


Fig. 3. Relationship between  $K$  values of arginase obtained with 3 per cent arginine carbonate and those obtained with 1 per cent arginine carbonate. O, values obtained without  $\text{MnSO}_4$ ;  $\Delta$ , values obtained with  $\text{MnSO}_4$ .

In determining the arginase activity of isolated rat liver nuclei, 0.1 ml. of a suspension containing about 8 to 10 mg. of nuclei (dry weight) per ml. was added to the arginine carbonate. In determining the activity of whole rat liver, 0.2 to 0.3 gm. of liver was homogenized in a ground glass homogenizer of the design shown in Fig. 4, with 5 ml. of water. 0.1 ml. of the suspension which contained 12 to 15 mg. (dry weight) per ml. was added to the arginine carbonate solution. The arginine carbonate was prepared by adjusting arginine monohydrochloride to pH 9.0 with sodium carbonate.

In working with tissues other than liver, more concentrated suspensions were used and in some cases (nuclei of kidney cells, pancreas cells, and chicken liver) the reaction was allowed to proceed for 1 to 3 hours. In cases in which  $\text{MnSO}_4$  was used as an activator, this was added as 0.1 ml. of a 1 per cent solution.

It should be pointed out that in all cases in which nuclei were isolated

from rat liver five to six livers were pooled to give 50 gm. of material from which the nuclei were obtained. Thus each value for the  $K$  per gm. of isolated nuclei of rat liver cells in a way represents an average value for five or six livers. In working with rat kidney, a much larger number than six kidneys was used. With dog kidney, however, the kidneys of only one animal were used. In the case of lamb and chicken, two pairs of kidneys were used.

Approximately equal numbers of male and female rats were used in determining the values of  $K$  per gm. of the whole liver homogenate and in making

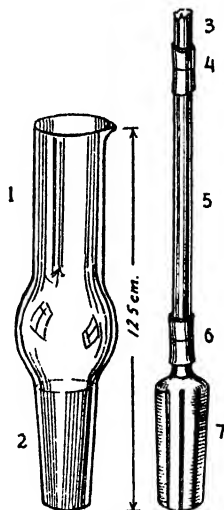


FIG. 4. Ground glass homogenizer. 1, Pyrex glass homogenizer tube; 2, bottom of homogenizer tube, ground to fit the pestle; 3, short glass rod held in the chuck of the stirring motor; 4, rubber or Tygon connecting tubing; 5, Pyrex glass rod; 6, rubber or Tygon connecting tubing; 7, pestle ground to fit the bottom of the homogenizer tube, with grooves on side and bottom.

a given sample of nuclei, but in general no attention was paid to the sex of the animal in a given determination.

*Analysis of Isolated Nuclei from Liver Cells for Metals*—A sample of rat liver cell nuclei of 86 mg. of dry weight was ashed in the muffle furnace and the ash, which weighed 1 mg., was digested with a small amount of concentrated  $H_2SO_4$ . Most of the  $H_2SO_4$  was then gradually replaced by  $HCl$  with evaporations nearly to dryness, and finally the residue was taken up in 2 ml. of distilled water. A small amount of material which remained undissolved and which probably contained a high percentage of silica was discarded, and the supernatant solution was analyzed spectrographically for the metals by Dr. L. T. Stedman of the Atomic Energy

Project (see Table III). The values in Table III are calculated on the basis of gm. of the element per 100 gm. of nuclei (dry weight).

*Results of Determination of Arginase Activities of Various Whole Tissues and Isolated Cell Nuclei*—Table I includes a number of determinations of arginase in whole tissue homogenates from rat and hen liver and dog, lamb, rat, and hen kidney. The results of determinations of the arginase activities of cell nuclei isolated from these tissues also are indicated. The distribution of the values for arginase activity in normal rat liver homogenate, determined on fifteen different liver samples without the addition of manganese, is shown in Fig. 5.

TABLE I

*Activity of Arginase Expressed As  $K^*$  Per Gm. of Dry Weight for Various Whole Tissue Homogenates and Isolated Cell Nuclei*

Type of material studied	Normal rat liver†	Regenerating rat liver†	Hen liver	Rat kidney	Dog kidney	Lamb kidney	Hen kidney	Sheep pancreas
Whole tissue	4.9 (15)	3.7 (1)	0	0.09 0.13	Trace	0.029	0.41	0
Isolated cell nuclei	7.6‡ (6)	5.4‡ (1)		0.11 Average				
	8.7	10.1	0	0	0	0	0	0
	9.2							
	8.9							
	7.4							
	8.6 Average							

\*  $K = 1/t \log_{10} A/(A - x)$ , where  $t$  = reaction time in minutes,  $A$  = mg. of dioxanthyl urea that would be formed from complete hydrolysis of substrate, and  $x$  = mg. of dioxanthyl urea found in experiment.

† The figures in parentheses denote the number of experiments.

‡  $\text{MnSO}_4$  added as an activator; all other results are without the addition of  $\text{MnSO}_4$ .

The data in Table II show that the arginase of whole liver is not fully activated with manganese, since the addition of manganous sulfate increases the activity very markedly. In most of these experiments 0.1 ml. of 1.0 per cent  $\text{MnSO}_4$  was added to the 1 ml. of arginine carbonate used for substrate, prior to addition of the whole tissue or nuclei.

The addition of manganous sulfate to isolated liver cell nuclei on the other hand does not increase their arginase activity at all. This is true even when the nuclei are allowed to remain in dilute  $\text{MnSO}_4$  solution before adding them to the substrate. It seems likely that the arginase of the cell nuclei is quite well liberated from the nuclear residue at pH 9.0 in arginine car-

bonate; therefore it appears probable that there is sufficient metal in the isolated nuclei to activate the enzyme completely, although this is not true for whole liver homogenate.

If the assumption is made that isolated cell nuclei are about 70 per cent water, it can be calculated from data given in Table II that the manganese concentration in isolated liver cell nuclei is approximately  $6 \times 10^{-5}$  M.

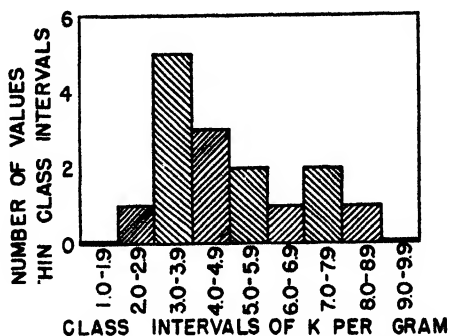


FIG. 5. Distribution of values for arginase activity of normal rat liver homogenates not activated with  $\text{MnSO}_4$ , expressed as values of  $K$  per gm. of homogenate (dry weight).

TABLE II

*Effect of Manganous Sulfate in Activating Arginase in Whole Rat Liver Homogenate*

The values listed in each row across were obtained on the same liver homogenate.

Without $\text{MnSO}_4$	With $\text{MnSO}_4$	Per cent activation
<i>K per gm.</i>	<i>K per gm.</i>	
5.2	6.4	24
4.4	6.7	52
4.3	6.9	63
3.4	5.5	64
3.0	4.1	35
3.8	7.5	96
Average 4.0	6.2	56

This concentration of manganese might be sufficient for activation of all of the arginase in the nuclei even if none of the other metals which are listed in Table III as being present in the nuclei contribute anything to the metal activation of arginase.

The arginase activity of isolated rat liver cell nuclei decays rapidly for a time and then remains at approximately a constant activity when the nuclei are kept at  $+3^\circ$  in the ice box, suspended in distilled water. Decay curves

for three samples of nuclei are shown in Fig. 6. These curves show that the nuclei must be freshly made if maximal arginase activity is to be obtained, and also that manganese does not affect the decay curve appreciably. The values listed in Table I for the arginase activities of isolated cell nuclei are for freshly prepared nuclei, not more than 6 hours old if the time is counted

TABLE III  
*Metals in Isolated Cell Nuclei of Rat Liver*

Element	Per cent in rat liver cell nuclei
Cu.....	$2.5 \times 10^{-3}$
Al.....	$1.7 \times 10^{-3}$
Fe.....	$1.1 \times 10^{-3}$
Mg.....	$7.0 \times 10^{-3}$
Mn.....	$1.4 \times 10^{-3}$
Zn.....	$1.2 \times 10^{-3}$
Ca.....	$2.3 \times 10^{-4}$

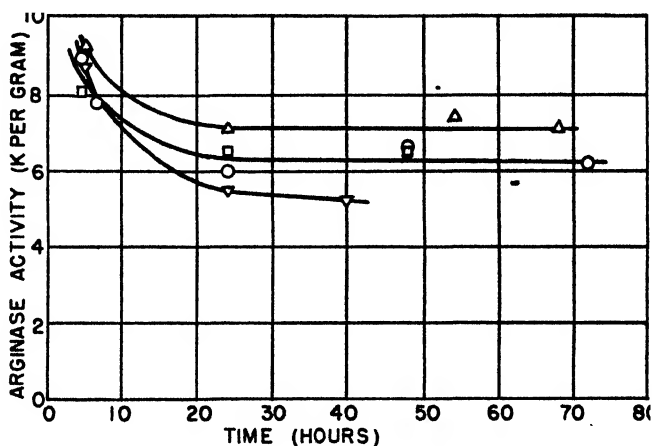


FIG. 6. Decay of arginase in isolated cell nuclei preparations. ▽, Sample 1, without  $\text{MnSO}_4$ ; ○, Sample 2, without  $\text{MnSO}_4$ ; □, Sample 2, with  $\text{MnSO}_4$ ; △, Sample 3, without  $\text{MnSO}_4$ .

from the moment the frozen liver is added to the Waring blender. It is assumed that the arginase in the cell nuclei does not decay appreciably before the isolation of the nuclei while the liver is being frozen, since this appears to be true for the whole tissue.

The results given in Table I show that the average value for  $K$  per gm. of cell nuclei isolated from normal rat liver is 8.6, while the average value of  $K$  per gm. of whole normal rat liver homogenate, after activation with

$\text{MnSO}_4$ , is 7.6. This latter value was calculated by applying the average per cent activation caused by  $\text{MnSO}_4$  in the six experiments shown in Table II to the average value of the arginase  $K$  per gm. for whole rat liver homogenate given in Table I and calculated from the fifteen determinations on different liver samples for each determination. (This average value is 4.9;  $(4.9 \times 0.56) + 4.9 = 7.6$ .) The value of  $K$  per gm. of 7.6, thus obtained, is higher than the average value of 6.2 obtained directly from the data in Table II, but the values of  $K$  per gm. without adding  $\text{MnSO}_4$ , which are given in Table II, yield an average value which is considerably lower than that obtained by averaging all of the fifteen determinations.

Briefly then, the average  $K$  per gm. of arginase in cell nuclei of rat liver is about 113 per cent of the value of completely activated whole rat liver homogenate; the values of  $K$  per gm. for dog, lamb, rat, and chicken kidney are very much lower, and the value of  $K$  per gm. of cell nuclei isolated from the above tissues is negligibly small compared to that of rat liver or cell nuclei of rat liver.

No attempt was made to determine whether the trace of precipitate obtained with cell nuclei of the latter tissues was due to a very small but real arginase activity or whether it was an artifact due, for example, to bacterial action. We can state that  $K$  per gm. of these nuclei was at the most not greater than 0.002, or 10 per cent of the average value for lamb kidney. The time of incubation required to get enough urea to give any visible dioxanthyl urea precipitate was so long that bacterial growth must have occurred in at least some cases. The values for the whole kidney homogenates represent real values for arginase activity, as anyone acquainted with the literature on the determination of arginase will no doubt concede, but we do not claim that our method of determination gives very accurate results in the case of kidney homogenates in which the arginase activity is quite low.

*Experiments Relating to Possible Adsorption of Cytoplasmic Arginase by Cell Nuclei*—A quantity of nuclei isolated from dog kidney and calculated to weigh 18.6 mg., if dried, was centrifuged from an aqueous suspension and was resuspended in 2 ml. of partially purified, soluble beef liver arginase solution having a monomolecular reaction velocity constant  $K$  of 2.2 per ml. (This represents a fairly strong arginase solution.) The arginase solution, which was water-clear and nearly colorless, and which contained a small amount of  $\text{MnSO}_4$  as activator, had been prepared previously from beef liver.

The nuclei were recovered by centrifugation from the arginase solution after having been suspended in it for half an hour and were washed six times with distilled water. The  $K$  per gm. (dry weight) of the recovered nuclei was 0.11. By referring to Table I it can be seen that the nuclei had gained

an appreciable amount of arginase activity, but on a dry weight basis their activity was only 1.3 per cent of the activity of nuclei isolated from rat liver cells.

This experiment was repeated, with more dilute beef liver arginase, in order to duplicate more closely the arginase activity of the supernatant of rat liver homogenate used for isolation of cell nuclei. A quantity of nuclei from lamb kidney calculated to weigh 4 mg., if dried, was suspended for half an hour in 1 ml. of arginase solution having a monomolecular reaction velocity constant  $K$  of 0.03 per ml. The nuclei were recovered by centrifugation, washed six times with distilled water, and the arginase concentration was determined. The  $K$  per gm. of nuclei, on a dry weight basis, was 0.28, which indicates that the nuclei had an activity of only 3.3 per cent that of cell nuclei of rat liver, on a dry weight basis.

Another experiment was carried out in which the arginase was added to the homogenizing material in the Waring blender 1 minute before homogenization was stopped. In this case the arginase concentration was adjusted so as to be of the same order of magnitude as the concentration of arginase in the clear supernatant from the rat liver homogenate after centrifuging off both the nuclei and the insoluble cell granules in a high speed centrifuge. This procedure was adopted because it was found that much of the cytoplasmic arginase in the case of liver remained bound to cytoplasmic granules at the pH of the isolation of nuclei (6.0). This bound portion of the arginase therefore was discarded when the nuclei were freed from the last contaminating granules.

After adding the arginase to the suspension of lamb kidney in the Waring blender, the lamb kidney nuclei were isolated as usual, and their arginase activity was measured. Again appreciable arginase had combined with the nuclei; its concentration in the nuclei amounted to  $K$  per gm. of 0.52. This is, however, only 6 per cent of the arginase activity of nuclei isolated from rat liver.

Finally the experiment described last was repeated exactly, except that chicken liver was used instead of lamb kidney. In this case no detectable amount of arginase appeared in the isolated nuclei.

The results of the experiments on the possibility of adsorption of arginase by cell nuclei from the cytoplasmic arginase are summarized in Table IV. If cell nuclei of rat liver behave similarly to cell nuclei of dog and lamb kidney and chicken liver, it is unlikely that a transfer of dissolved enzyme from the cytoplasmic material to the nuclei during isolation of the nuclei could be of sufficient magnitude to affect the quantity of arginase in the nuclei appreciably. It also appears unlikely that lamb arginase would behave in a significantly different manner towards lamb cell nuclei than would beef arginase. If crystalline arginase should become available in the



future, however, it might be worth while to carry out an experiment on "adsorption" of arginase by nuclei with the homologous enzyme.

**Possible Adsorption of Arginase by Cell Granules**—It may be of interest to those working on isolated cell granules to know that the amount of added arginase taken up or adsorbed by the cytoplasmic granules was negligible in the experiment in which the diluted beef liver arginase was added to the homogenized lamb kidney in the Waring blender. In this work a sample of the granules (all sizes taken together) was obtained by centrifugation in the cold at 18,000 R.P.M. The supernatant solution, which was perfectly clear, contained practically all of the added arginase.

TABLE IV  
*Adsorption of Arginase by Cell Nuclei*

	Isolated cell nuclei placed in arginase solution		Arginase solution added to cell homogenate in Waring blender; nuclei then isolated	
	Cell nuclei of dog kidney with strong arginase solution, K per ml. = 2.2	Cell nuclei of dog kidney with diluted arginase solution, K per ml. = 0.03	Cell nuclei of lamb kidney	Cell nuclei of chicken liver
Arginase activity, K per gm. dry weight	0.11	0.28	0.52	0
Arginase activity, % of activity of rat liver nuclei	1.3	3.3	6.0	0

#### DISCUSSION

It has been demonstrated in the foregoing paragraphs that the arginase content of the nuclei of rat liver cells is in all probability as high or higher in concentration than it is in the cytoplasm. The studies on whole liver can be taken to approximate cytoplasm, since the amount of nuclear material in a whole liver homogenate cannot be more than 10 per cent on a dry weight basis, as can be deduced from the studies of Marshak (4). One of the simplest possible explanations of this finding is that the enzyme arginase is synthesized in the cell nucleus and then passes out into the cytoplasm. It appears to us that the arginase located in the liver cell nucleus is not likely to have any functional significance peculiar to the cell nucleus, since this enzyme appears to fulfil a special rôle in metabolism in the formation of urea which in animals is limited to the liver. Furthermore, the substrates for urea formation must to a large extent come into the liver cells from the circulation, and thus they have to reach the cytoplasm before penetrating to the nucleus. It is entirely possible that the concentration of arginine in the nucleus may for this reason be very low compared to its concentration in cytoplasm where presumably the bulk of the urea formation is occurring.

For this reason the nuclear arginase may have little or no substrate to act upon.

It has also been demonstrated that the arginase activity of nuclei isolated from kidney cells is negligible, although arginase activity is easily demonstrable in whole kidney tissue.

It has been suggested by Kochakian (5) that the arginase of kidney is in reality a transamidinase which is responsible for the formation of glycoamine and which accidentally possesses weak arginase activity. The fact that liver arginase is enormously higher in activity than kidney arginase lends strength to this idea. It is hard to imagine that such a small amount of arginase as occurs in the kidney can have any functional significance unless it is concentrated in a limited number of cells. If indeed the so called kidney arginase is not a true arginase, there is no reason *a priori* to predict its presence in kidney cell nuclei, since even in the case of liver we have encountered enzymes such as choline oxidase (6) and succinic dehydrogenase<sup>3</sup> (2) which are present in very low concentration or absent in the cell nuclei.

The chief findings of interest in this work are, therefore, that the enzyme arginase appears to be a real constituent of nuclei of rat liver cells, and that it is present there in as high or higher concentration than in the cytoplasm. The latter finding indicates the possibility that arginase may be synthesized in the cell nucleus, particularly when one realizes that there may be little or no substrate for the enzyme to work upon in the nucleus.

#### SUMMARY

1. Cell nuclei have been isolated by an improved method from normal rat liver, normal chicken liver, normal dog, lamb, rat, and chicken kidney, and normal sheep pancreas, and the arginase activities of the cell nuclei isolated from these tissues have been compared.

2. The arginase activity per dry weight of cell nuclei isolated from normal rat liver is now found to be about 113 per cent of the activity of normal whole rat liver homogenate when the latter is activated by the addition of  $\text{MnSO}_4$ .  $\text{MnSO}_4$  does not increase the arginase activity of nuclei isolated from normal rat liver.

<sup>3</sup> Recent repetition of work on succinic dehydrogenase with cell nuclei of rat liver isolated by the improved method (3) has confirmed our earlier results (2) that the activity of this enzyme is negligible in the nuclei compared to its activity in the whole liver homogenate. Both the Warburg technique and the methylene blue technique again were used. As before (2) cytochrome oxidase was found to be present in very appreciable amount, although a careful reinvestigation of its quantitative activity has not yet been made. We wish to thank Frederick G. Smith for estimating the cytochrome oxidase activity of the isolated nuclei by means of a new colorimetric method. It thus appears to be possible to use the absence of succinic dehydrogenase as one indication of freedom from cytoplasm of the isolated cell nuclei.

3. The arginase activities of cell nuclei isolated from the kidney of dog, lamb, rat, and chicken are extremely small or zero, although by the method employed the presence of arginase could easily be shown in the whole homogenates of these tissues, all of which have low arginase activities compared to normal rat liver homogenates.

4. Experiments designed to detect a possible transfer of arginase from the soluble cytoplasmic fraction to the nuclei during the process of isolation of the nuclei indicate that any such transfer would probably be too small in magnitude to affect appreciably the arginase activity of the isolated nuclei. The same can be said, incidentally, of a possible transfer of dissolved arginase to the cytoplasmic granules.

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# EFFECT OF AMINO ACIDS ON THE GROWTH OF RATS ON NIACIN-TRYPTOPHAN-DEFICIENT RATIONS\*

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Previous work (1, 2) in our laboratory showed that the addition of protein hydrolysates or certain amino acids to a 9 per cent casein-sucrose ration deficient in niacin resulted in a marked reduction in the growth rate of rats. The further addition of either niacin or tryptophan restored growth to a rate equal to or exceeding that of rats receiving the unsupplemented basal ration. Subsequent experiments (3) have shown that a mixture of amino acids simulating 2 per cent acid-hydrolyzed casein is equally effective in inducing the niacin deficiency. The experiments reported in this paper deal with the effect of individual amino acids and the possible mechanism of their action.

## EXPERIMENTAL

The procedures used in these experiments were similar to those employed in previous studies. 3 week-old male Sprague-Dawley rats, kept in individual cages, were fed *ad libitum* over a 5 week experimental period, and weighed at weekly intervals. The basal diet had the following composition: sucrose 81.8 per cent, corn oil 5 per cent, casein<sup>1</sup> 9 per cent, Salts IV 4 per cent (4), L-cystine 0.2 per cent, and vitamins added as a dry mixture diluted with sucrose to provide the following quantities per 100 gm. of diet: thiamine 0.2 mg., riboflavin 0.3 mg., pyridoxine 0.25 mg., calcium DL-pantothenate 2 mg., choline chloride 100 mg., inositol 10 mg., biotin 0.01 mg., and folic acid 0.02 mg. Halibut liver oil fortified with vitamins E and K and diluted with corn oil was used as a source of the fat-soluble vitamins. This was fed by dropper once each week to provide approximately the fol-

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<sup>1</sup> Smaco purified casein.

lowing daily intakes: vitamin A 400 i.u.,<sup>2</sup> vitamin D 4 i.u., 2-methyl-1,4-naphthoquinone 0.06 mg., and  $\alpha$ -tocopherol 0.7 mg. The average growth rate for twelve negative control rats was 12 gm. per week, and for twelve positive control animals 16 gm. per week in the series of four experiments reported here. The symptoms noted in those animals which received the rations containing growth-suppressing amino acids were roughness of fur and inflammation of the paws and nose. These symptoms appeared after the rats had received the experimental ration for more than 2 weeks.

Feces were collected from selected groups of animals during the 4th week in two of the experiments. The feces were dried *in vacuo*, and samples weighing 0.4 gm. were hydrolyzed with 20 cc. of 1 N NaOH by autoclaving at 15 pounds pressure for 1 hour. After neutralizing, the diluted samples were assayed for nicotinic acid according to the method of Teply *et al.* (5). The values obtained are recorded in Table III.

Following the 5 week growth period of one experiment urine was collected under toluene from rats from selected groups. The N<sup>1</sup>-methylnicotinamide content was measured by the acetone-fluorometric method of Huff *et al.* (6).

In the first experiment two mixtures of amino acids were used; Mixture I contained those amino acids which had been fed in the previous experiment as the naturally occurring L isomers, and Mixture II those which had been fed as the racemic mixture. These mixtures represented an arbitrary division of the amino acids previously used (3) into two groups based on the isomeric forms most readily available commercially. These amino acids were added to a 9 per cent casein ration at 1.267 per cent of Mixture I or 1.046 per cent of Mixture II to give the same concentrations of individual amino acids as previously used.

#### RESULTS AND DISCUSSION

The results of studies on the effect of amino acids on the growth of rats receiving the basal ration with and without niacin are shown in Table I. The growth rates are tabulated as percentage of the growth of the negative control group or of the positive control group. This procedure and the criteria used in evaluating the results are the same as those previously employed (3). The addition of amino acid Mixture II resulted in a very marked inhibition of growth (14 per cent of the growth of negative controls), while Mixture I was only slightly effective in suppressing growth (72 per cent of negative controls).

A second experiment involved the use of the individual amino acids in Mixture II. Each of these amino acids was added separately to a 9 per

<sup>2</sup> In the preceding paper (3) of this series the vitamin A and D content was erroneously reported as vitamin A 6 i.u. and vitamin D 0.06 i.u. The levels should be the same as given in this paper.

TABLE I

*Effect of Certain Supplements on Growth Rate of Rats Receiving Low Niacin Diets*

Supplement	Without niacin or tryptophan		Plus 1.5 mg. niacin per 100 gm. ration	
	No. of animals	Average gain as per cent of negative controls	No. of animals	Average gain as per cent of positive controls
2% acid-hydrolyzed casein ( $H_2SO_4$ )	6	43	3	119
2% " " (HCl) "	6	64		
1.267% amino acids (Mixture I)*	3	72	3	77
1.046% " " ( " II)†	3	14	3	115
2.534% " " ( " I)	3	100	3	119
0.104% DL-phenylalanine	12	57	3	110
0.208% " "	3	51		
0.052% " "	3	79		
0.104% D-phenylalanine	3	58		
0.078% DL-threonine	3	21		
0.156% " "	12	17	3	125
0.156% " " + 0.05% DL-tryptophan (no niacin)			3	168
0.104% DL-phenylalanine + 0.156% DL-threonine	3	22		
DL-Threonine (injected) 15.6 mg. per day	3	33	3	137
DL-Phenylalanine (injected) 10.4 mg. per day	3	117	3	106
0.126% DL-aspartic acid	3	91		
0.28% DL-valine	3	100		
0.26% DL-isoleucine	3	91		
0.52% " "	3	72		
0.12% DL-serine	3	104		
0.164% L-arginine hydrochloride	3	75		
0.1% L-histidine hydrochloride·H <sub>2</sub> O	3	83		
0.2% " " "	3	58		
0.256% L-tyrosine	3	117		
0.276% L-lysine hydrochloride·H <sub>2</sub> O	3	87		
0.912% L-glutamic acid	3	115		
0.484% L-leucine	3	103		
0.328% L-proline	3	71		
0.5% fat-isocaloric replacement of 4.5% corn oil by sucrose	6	71	6	107
30% fat-isocaloric replacement of sucrose by 25 gm. corn oil	6	108	6	131
2% glycine	9	62		
2% " " + 0.5% fat-isocaloric replacement of 4.5% corn oil by sucrose	6	33		
2% glycine + 30% fat-isocaloric replacement of sucrose by 25 gm. corn oil	6	92		
3.12% N-ethylglycine	3	40		

TABLE I—*Concluded*

Supplement	Without niacin or tryptophan		Plus 1.5 mg. niacin per 100 gm. ration	
	No. of animals	Average gain as per cent of negative controls	No. of animals	Average gain as per cent of positive controls
3.72% glycine ethyl ester	3	17		
2% glycine + 1 mg. pyridoxine	3	40		
0.156% DL-threonine + 1 mg. pyridoxine	3	17		
0.104% DL-phenylalanine + 1 mg. pyridoxine	3	66		
2% sulfasuxidine	3	75		
2% " + 0.104% DL-phenylalanine	3	83		
2% sulfasuxidine + 0.156% DL-threonine	3	25		

\* Amino acid Mixture I: L-arginine hydrochloride 0.082 gm., L-histidine hydrochloride·H<sub>2</sub>O 0.05 gm., L-lysine hydrochloride·H<sub>2</sub>O 0.138 gm., L-tyrosine 0.128 gm., L-leucine 0.242 gm., L-glutamic acid 0.456 gm., and L-proline 0.164 gm.

† Amino acid Mixture II: DL-phenylalanine 0.104 gm., DL-serine 0.12 gm., DL-threonine 0.156 gm., DL-isoleucine 0.26 gm., DL-aspartic acid 0.126 gm., and DL-valine 0.28 gm. All of the amino acids in these mixtures are at the same level as in 2 per cent acid-hydrolyzed casein with the exception of threonine and isoleucine, which were added in quantities equal to twice those present in this amount of casein hydrolysate. Alanine, glycine, and cystine were left out of the mixtures because the growth inhibition effect of the first two is known, and cystine was incorporated into the basal ration.

cent casein ration in amounts equivalent to their occurrence in 2 per cent acid-hydrolyzed casein. It was found that 0.078 per cent DL-threonine suppressed growth markedly (17 per cent growth of negative controls) and 0.104 per cent DL-phenylalanine, though fulfilling the criteria adopted, was not as effective as DL-threonine. In both cases the addition of niacin (1.5 mg. per 100 gm.) to these diets resulted in growth rates greater than those for positive control groups. When 50 mg. of DL-tryptophan per 100 gm. were added to the DL-threonine-supplemented ration, a greater increase in the growth rate was noted (125 per cent with added niacin, 168 per cent with added tryptophan. The increased growth resulting from the addition of DL-threonine to the basal ration containing added cystine and niacin or tryptophan indicates that threonine may be the next most limiting nutrient in this ration. Hall and Sydenstricker (7) have shown that threonine is one of five amino acids which are limiting in a 9 per cent casein basal ration containing added cystine. Additional histidine, lysine, valine, threonine, and tryptophan were necessary for optimal growth.

In another experiment the L-amino acids in Mixture I were investigated individually at twice the levels present in 2 per cent acid-hydrolyzed casein.

None of the L-amino acids suppressed growth sufficiently to meet the criteria adopted. However, it was noted that the incorporation of L-histidine at 0.2 per cent (4 times the level present in Mixture I) of the ration caused growth suppression to 58 per cent of that of the negative control group.

The succeeding experiments were conducted in an effort to elucidate the mechanism of the inhibition of growth by DL-threonine and DL-phenylalanine. The D isomer of phenylalanine<sup>3</sup> was found to be as effective as the racemic acid when fed to one group of three animals. Further work is now in progress to determine the relative activity of the D and L forms. To compare two routes of administration of threonine and phenylalanine two groups of three rats each were injected with amounts of these amino acids approximately equal to those received in the ration by control rats. Solutions containing 15.6 mg. per ml. of DL-threonine or 10.4 mg. per ml. of DL-phenylalanine were injected in three equal doses over a 24 hour period. DL-Threonine gave marked growth suppression whether injected or fed, and this suppression was overcome by the addition of niacin to the ration. The ineffectiveness of 10.4 mg. per day of DL-phenylalanine intraperitoneally may be the result of too small a quantity for this route of administration. Failure to obtain an additive effect from 0.156 per cent DL-threonine and 0.104 per cent DL-phenylalanine (Table I) may have been the result of too much threonine or insufficient phenylalanine or both to demonstrate such an effect. At equal levels of oral intake threonine is far more effective than phenylalanine.

That the intestinal microflora may be playing a rôle in the influence of these individual amino acids on growth is shown by the fact that, when DL-threonine or DL-phenylalanine was added to a ration containing dextrin instead of sucrose, the growth inhibition was not marked (Table II). The incorporation of gelatin at a level of 6 per cent caused growth inhibition, indicating that two separate mechanisms may be involved in the suppression caused by gelatin and threonine. Further, the growth failure when gelatin was fed and the correction by niacin indicates that the niacin present in the dextrin was not responsible for this carbohydrate difference. Acid-hydrolyzed casein gave only slight growth depression when added to a 9 per cent casein-dextrin ration. The amount of inhibition obtained with acid-hydrolyzed casein when sucrose was the carbohydrate seemed to be affected by the method of hydrolysis used. A sulfuric acid hydrolysate<sup>4</sup>

<sup>3</sup> We are indebted to Mr. D. G. Doherty for D-phenylalanine.

<sup>4</sup> Smaco casein was hydrolyzed with 5 volumes of 5 N H<sub>2</sub>SO<sub>4</sub> for 16 hours at 15 pounds pressure in the autoclave and neutralized with solid barium hydroxide. The combined filtrate and washings were concentrated *in vacuo* to a syrup which was dried to a porous cake in a vacuum oven at 50°. The mixture was finely powdered in a mortar and stored in a brown bottle until it was incorporated into the dry ration. The mixture was light amber and very hygroscopic.



gave stronger inhibition than a hydrochloric acid hydrolysate<sup>5</sup> (Table I). The determining factor may have been the threonine and phenylalanine content of the hydrolysates, since microbiological determinations showed that 2 per cent of the sulfuric acid hydrolysate provided phenylalanine at 0.072 per cent of the ration and threonine at 0.055 per cent. 2 per cent of the hydrochloric acid hydrolysate furnished phenylalanine at 0.020 and threonine at 0.044 per cent of the ration.

Other factors such as vitamin and fat levels were investigated. Schweigert *et al.* (8), Rosen *et al.* (9), and Bell *et al.* (10) have shown that the pyridoxine intake influences the quantities of niacin derivatives in the urine. In these experiments increasing the pyridoxine level from 0.25 to 1 mg. per 100 gm. of diet did not counteract the growth inhibition caused by free amino acids (Table I). Salmon (11) noted poor growth in rats fed

TABLE II  
*Effect of Certain Supplements When Ration Contains Dextrin*

Supplement	Without niacin		Plus 1.5 mg. niacin per 100 gm. ration	
	No. of animals	Average gain as per cent of negative controls	No. of animals	Average gain as per cent of positive controls
2% acid-hydrolyzed casein.....	6	65	3	92
6% gelatin.....	3	15	3	103
0.156% DL-threonine.....	3	80		
0.104% DL-phenylalanine.....	3	82		
6% gelatin plus 30% fat.....	3	29		

diets low in fat containing up to 18 per cent casein, and no known source of a deleterious agent. He found that when the fat level was increased to 30 per cent the niacin deficiency was relieved. Qualitatively the same results were obtained in our studies when the level of fat in the ration was varied from 0.5 to 30 per cent. The reduction of the fat level to 0.5 per cent gave a slight reduction in growth, and elevation of the fat level to 30 per cent gave a very slight increase in growth compared to 5 per cent corn oil (Table I). The effect of varying the level of fat on the growth of rats receiving the basal ration containing 2 per cent glycine (2) was also in-

<sup>5</sup> Smaco casein was hydrolyzed with 5 volumes of 5 N HCl for 16 hours at 15 pounds pressure in the autoclave. The solution was filtered, and concentrated *in vacuo* to a syrup. Water was added and the solution concentrated again to a syrup. This process was repeated until the solution was approximately pH 3. The hydrolysate from 1 kilo of casein in 3 liters of hot solution was treated with 300 gm. of norit and filtered through a fluted filter. After concentrating, it was dried to a porous cake in a vacuum oven at 50°. The cake was powdered and stored in a brown bottle.

vestigated. The addition of 30 per cent fat to the ration relieved the growth inhibition of 2 per cent glycine, while the reduction of the fat level to 0.5 per cent reinforced the inhibition. Better growth with high fat rations may be explained on the basis of a sparing action on the niacin as suggested by Salmon (11), or by synthesis or decreased destruction of this vitamin by intestinal microorganisms when a high fat ration is fed.

Derivatives of glycine were tested on the 9 per cent casein basal ration. Two groups of animals were fed the ration containing 3.12 per cent N-ethyl glycine or 3.72 per cent glycine ethyl ester. Both derivatives gave a slightly stronger inhibition than an equivalent amount (2 per cent) of glycine itself.

The analyses of the dried feces for nicotinic acid revealed that the daily excretion of this vitamin was approximately the same (7 to 12  $\gamma$  per day) in all groups regardless of the body weight or quantity of feces excreted. When expressed on the basis of dry weight of feces or per 100 gm. of body weight per day (Table III), the animals whose growth was poor showed greater niacin excretion, regardless of the ration received. This relationship was markedly altered, however, by the presence of sulfasuxidine, which reduced the excretion from more than 100  $\gamma$  per gm. to 10  $\gamma$  per gm., when added to the threonine-supplemented ration, from 40 to 10  $\gamma$  per gm. for the unsupplemented basal ration, and from 50 to 10  $\gamma$  per gm. for phenylalanine-supplemented animals. It is interesting that the sulfasuxidine which exerted such a profound effect on the niacin content of feces had no effect on the growth rate of the animals. This finding as well as the inverse relationship between feces niacin and growth suggests that the niacin synthesis in the lower tract is not the determining process in the nutritional relationships being studied. That niacin synthesis in some portion of the tract is of some importance, however, is suggested by the effect of substituting dextrin for sucrose in the diet. As previously reported (5), this carbohydrate greatly elevated niacin excretion in the feces. Regardless of the supplements fed, rats receiving dextrin excreted approximately 130  $\gamma$  per gm. or 50  $\gamma$  per 100 gm. of body weight per day.

The results of the measurements of urinary N<sup>1</sup>-methylnicotinamide indicated that there was no significant effect of any supplement except tryptophan. In this group urinary excretion was 87  $\gamma$  per day per 100 gm. of body weight compared to 7.0  $\gamma$  for control animals. Urinary excretion of this metabolite was elevated in rats receiving the dextrin-containing rations to 21  $\gamma$  per day. No significant difference was found in the threonine and tryptophan content of protein-free filtrates of liver and plasma with the microbiological assay. Growth inhibition of an amino acid mixture containing 0.25 per cent L-histidine, 0.4 per cent DL-threonine, 0.52 per cent L-lysine, and 0.3 per cent DL-valine has been reported by Singal *et al.* (12),

TABLE III  
Niacin Excretion in Feces

Experiment No.	Supplement	Feces		Body weight at collection	Body weight	
		Average	Range	Average	Average	Range
		$\gamma$ per gm.	$\gamma$ per gm.	gm.	$\gamma$ per day per 100 gm.	$\gamma$ per day per 100 gm.
I	Sucrose basal	70	(42-109)	74	17	(11-21)
	" " + niacin	36	(30- 41)	95	7	(6- 8)
	Basal + acid-hydrolyzed casein	70	(45-100)	61	12	(7-21)
	" + 2% glycine	103	(94-111)	57	20	(17-21)
	" + 0.078% DL-threonine	100	(77-121)	56	19	(11-30)
	" + 0.156% "	82	(61-110)	53	14	(10-21)
	" + 0.208% DL-phenylalanine	62	(32-108)	67	13	(7-21)
	" + 0.104% "	49	(29- 79)	76	9	(7-10)
	" + 0.104% "	89	(83- 90)	60	16	(12-23)
	+ 0.078% DL-threonine					
	Basal + 0.156% DL-threonine + 0.05% DL-tryptophan	43	(36- 54)	117	9	(7-11)
	Dextrin basal	139	(110-166)	126	50	(38-68)
	" " + niacin	124	(112-137)	134	44	(36-54)
	" " + 0.156% DL-threonine	132	(120-161)	123	43	(24-54)
II	Dextrin basal + 0.104% DL-phenylalanine	149	(116-191)	115	48	(34-65)
	Sucrose basal	44	(44- 46)	79		
	" " + niacin	40	(32- 45)	101		
	Basal + 0.104% DL-phenylalanine + 2% sulfasuxidine	10	(10- 10)	78		
	Basal + 0.156% DL-threonine + 2% sulfasuxidine	10	(8- 13)	53		
	Basal + 2% sulfasuxidine	10	(8- 11)	78		
	" + 0.156% DL-threonine	141	(66-221)	41		
	" + 0.104% DL-phenylalanine	50	(46- 54)	71		
	" + DL-threonine (injected) 15.6 mg. per day	101	(67-142)	56		
	Basal + niacin + DL-threonine (injected) 15.6 mg. per day	53	(36- 81)	123		
	Basal + DL-phenylalanine (injected) 10.4 mg. per day	58	(31-105)	92		
	Basal + niacin + DL-phenylalanine (injected) 10.4 mg. per day	47	(43- 55)	106		

using a similar ration in rat experiments. They found that the amino acid mixture had no effect on the niacin content of livers, whereas administration of niacin increased the quantity of this vitamin in the liver.

## SUMMARY

Addition of DL-threonine or DL-phenylalanine in amounts equivalent to that present in 2 per cent acid-hydrolyzed casein aggravated a niacin-tryptophan deficiency in rats. All other amino acids fed at comparable levels were without effect.

DL-Threonine injected intraperitoneally at 15.6 mg. per day caused growth suppression equal to that obtained when this amino acid was fed at the same level. Injection of 10.4 mg. of DL-phenylalanine per day was without effect.

The growth inhibition produced by either DL-threonine or DL-phenylalanine was reversed by the addition of 1.5 mg. per cent of niacin, 50 mg. per cent of DL-tryptophan, or by replacing the sucrose in the diet with dextrin.

Studies on the niacin content of the feces showed that all animals excreted approximately the same amounts per day. When the niacin content of the feces was expressed on a body weight basis or per gm. of dry feces, the values varied inversely with the growth rate, regardless of the ration used. The addition of 2 per cent sulfasuxidine to the rations sharply reduced the niacin in feces without affecting the growth rate.

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# AN INTERMEDIATE IN THE BIOSYNTHESIS OF LYSINE IN *NEUROSPORA*\*

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Investigations by Doermann (1, 2) have provided evidence for the existence of at least four distinct genetic types of lysine-requiring mutants of *Neurospora*. One of these (strain 4545) has been utilized in a method for biological assay for this amino acid (3). More recently evidence for some interrelation between the biosynthesis of uridine and lysine (4) has prompted further investigations of the group of *lysineless* mutants.

The first substantial evidence concerning the mechanism of biosynthesis of lysine in *Neurospora* has been provided as a direct result of the recent discovery by Borsook and his associates (5, 6) that lysine is converted by mammalian tissue slices and homogenates to  $\alpha$ -amino adipic acid. It has now been found that one of the *Neurospora lysineless* mutants (strain 33933) is able to utilize  $\alpha$ -amino adipic acid in the place of lysine. Mutants of three other genetic types are unable to do so. Thus, evidence is provided that this 6-carbon dicarboxylic acid is a precursor in the biological synthesis of lysine by *Neurospora*. Since three of the genetically different mutants (strains 4545, 15069, and 37101) do not utilize  $\alpha$ -amino adipic acid, it is to be expected that the corresponding three genetic blocks are concerned with different steps in the conversion of  $\alpha$ -amino adipic acid to lysine. Consequently several compounds having a possible place in this series of reactions were tested for growth-promoting activity on all four mutants. These included the naturally occurring hydroxylysine (7),  $\alpha, \alpha'$ -diaminopimelic acid,  $\alpha$ -keto adipic acid, and 2-carboxy-6-piperidone.

Doermann (1, 2) has described a striking inhibition of the *lysineless* mutants by arginine and has attributed this effect to interference with the biological conversion of an external supply of lysine to utilizable natural intermediates in the mold. With  $\alpha$ -amino adipic acid as well as lysine to provide the growth substance for strain 33933, the inhibitory effects of arginine, glutamic acid, and asparagine have been investigated.

## EXPERIMENTAL

**Growth Requirements**—As reported by Doermann (1), *Neurospora* strains 4545, 15069, 33933, and 37101 require lysine for growth. Growth curves

\* This work was supported by funds from the Rockefeller Foundation.

for strain 33933 in the presence of L-lysine, L- $\alpha$ -aminoadipic acid, and DL- $\alpha$ -aminoadipic acid are given in Fig. 1. It will be noted that at high concentrations DL- $\alpha$ -aminoadipic acid is just as effective as the L isomer on a molar basis. In contrast it has been shown by Doermann (3) that the D isomer of lysine is not utilized appreciably in the presence of the natural L form.

The cyclized form of  $\alpha$ -aminoadipic acid (2-carboxy-6-piperidone) was also tested for growth promotion of strain 33933. Although the compound

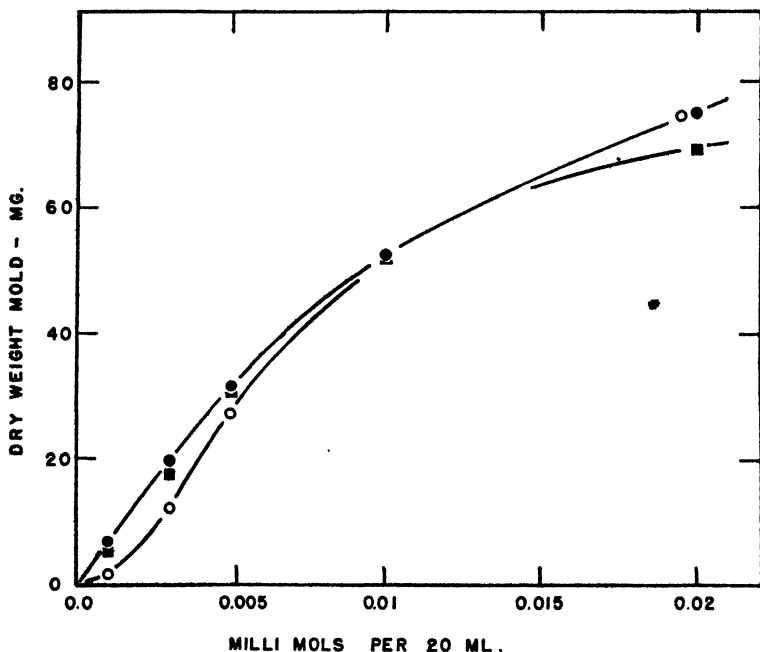


FIG. 1. Growth curves of mutant 33933 in the presence of L-lysine (●), L- $\alpha$ -aminoadipic acid (■), and DL- $\alpha$ -aminoadipic acid (○).

is not active in initiating growth, it is utilized completely in the presence of an equivalent amount of L- $\alpha$ -aminoadipic acid.

*Inactive Compounds*—Since D- $\alpha$ -aminoadipic acid is utilized by mutant strain 33933 in the presence of the L isomer, it would be expected that  $\alpha$ -ketoadipic acid would initiate and support growth of the mold. Similar cases have been reported previously in *Neurospora* (8, 9). Contrary to expectations, however, the keto acid was found to be without activity in initiating growth or supplementing growth initiated by an equal quantity of L- $\alpha$ -aminoadipic acid. Some results are presented in Table I. In the experiment given in Table I the keto acid solution, sterilized on a glass

filter, was added after 48 hours growth of the mold in the presence of the amino adipic acid. In other experiments, the keto acid failed to produce significant growth stimulation whether it was added after filter sterilization or as the sterile dry solid, either before or after initiation of growth.

TABLE I  
*Inactivity of  $\alpha$ -Ketoadipic Acid*

Substance added to 20 ml. of culture fluid		Dry weight of mold mg.
$\alpha$ -Ketoadipic acid mg.	L- $\alpha$ -Aminoadipic acid mg.	
1	0	0
3	0	0
0	1	41.0
0.5	1	40.5
1.5	1	43.0
3.0	1	40.0

TABLE II

*Inhibitions of Strain 33933 in Presence of Lysine and  $\alpha$ -Aminoadipic Acid*

All solutions contained 0.005 mm of growth substance per 20 ml. of culture fluid. 4 days growth.

Substance added		Dry weights of mold in presence of		
		L-Lysine	L- $\alpha$ -Aminoadipic acid	DL- $\alpha$ -Aminoadipic acid
	mm per 20 ml.	mg.	mg.	mg.
Arginine	0.0	32	32	27
	0.005	Trace	33	11
	0.01	0	27	10
	0.02	0	25	8
Glutamic acid	0.0	31.5	31	27
	0.005	31.5	32	29.5
	0.01	32.5	32.5	28
	0.02	31.5	33	14.5
Asparagine	0.0	31	30.5	30
	0.005	33	Trace	0
	0.01	34	0	0
	0.02	33.5	0	0

A preparation from gelatin containing hydroxylysine and lysine was generously furnished by Dr. Hiller and Dr. Van Slyke. 10 mg. of this material contained, as picrates, 1.7 mg. of lysine and a quantity of hydroxylysine equivalent to 1.9 mg. of lysine. Following removal of picric acid, quantitative assays for lysine with all four mutants gave values varying



from 1.55 to 1.65 mg. per 10 mg. of picrate. If hydroxylysine had the same molar activity as lysine for any of the mutants, a value of 3.6 should have been obtained. Since no significant loss of either amino acid occurred during the removal of the picric acid, it appears that hydroxylysine is not utilized by the *Neurospora* mutants.

$\alpha, \alpha'$ -Diaminopimelic acid was synthesized according to the method of Sørensen and Andersen (10). The compound did not promote growth of any of the four mutants and was, in fact, strongly inhibitory to growth in the presence of lysine. The compound has not been resolved.

*Growth Inhibitions*—Doermann (2) has reported inhibition of the lysine mutants by arginine and stimulation by asparagine and glutamic acid. These results were obtained in the presence of lysine as the required growth substance. In Table II the effects of these substances on strain 33933 in the presence of lysine and in the presence of amino adipic acid are compared.

As is shown in Table II, glutamic acid is not inhibitory to the utilization of L- $\alpha$ -amino adipic acid when the molar ratio is less than 4:1. When this ratio exceeds 10, however, glutamic acid does become inhibitory. Glutamine and aspartic acid have essentially the same effect as glutamic acid.

It should be noted that the inhibitory action of all of the compounds listed becomes less at high concentrations of  $\alpha$ -amino adipic acid or if tested over a long growth period. This is perhaps due to metabolism of the inhibitors by the mold.

#### DISCUSSION

It is apparent from the data presented that  $\alpha$ -amino adipic acid is a precursor of lysine in *Neurospora*. The utilization of D- $\alpha$ -amino adipic acid but not  $\alpha$ -keto adipic acid is an unexpected finding which suggests that the  $\alpha$ -amino group of amino adipic acid is metabolized in a unique fashion. Since this is known to be true of lysine, this result is perhaps not surprising, but it does necessitate further critical examination. The chemical identity of the preparation of the  $\alpha$ -keto adipic acid that was used appears to be beyond question.

The inhibition data obtained are quite in accord with the conclusions of Doermann (1). Whereas arginine is a potent inhibitor in the presence of lysine, it is relatively ineffective in the presence of L- $\alpha$ -amino adipic acid. Since Doermann's data provided evidence that arginine interfered with the conversion of lysine to some natural intermediate in the mold, the utilization of a precursor of such an intermediate should be relatively unaffected by arginine, as was found. It is to be noted, however, that arginine strongly inhibits the utilization of D- $\alpha$ -amino adipic acid. Asparagine and glutamic acid are also effective in this connection. The potent action of asparagine in the inhibition of utilization of L- $\alpha$ -amino adipic acid is sug-

gestive of the possibility that amide formation may occur in the conversion of  $\alpha$ -aminoadipic acid to lysine. This possibility is being investigated.

The authors wish to express their appreciation for the generous cooperation of Dr. H. Borsook and Dr. P. H. Lowy of this department. These investigators supplied the L- and DL- $\alpha$ -aminoadipic acids, the 2-carboxy-6-piperidone, and the  $\alpha$ -ketoadipic acid that were used in these investigations.

A preparation of hydroxylysine was obtained through the kindness of Dr. Hiller and Dr. Van Slyke of The Rockefeller Institute for Medical Research.

#### SUMMARY

1. Evidence is presented to show that  $\alpha$ -aminoadipic acid is a precursor of lysine for *Neurospora*.
2. Mutant 33933, which utilizes lysine or  $\alpha$ -aminoadipic acid, is inhibited by arginine in the presence of lysine and by asparagine in the presence of  $\alpha$ -aminoadipic acid. The significance of these inhibitions is discussed and conclusions are in accord with those previously set forward by Doermann.
3. It is shown that, although one mutant utilizes D- $\alpha$ -aminoadipic acid in the presence of the L isomer,  $\alpha$ -ketoadipic acid does not promote growth.

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# ON THE FAILURE OF DL-HOMOSERINE TO SUPPORT GROWTH ON A METHIONINE-DEFICIENT DIET\*

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This laboratory is concerned with certain problems involving the chemistry and the metabolic behavior of the hydroxyamino acids. In an earlier publication (1) the resolution of DL-homoserine has been described. The present report considers the question of whether homoserine can provide the carbon chain for the synthesis of methionine in the white rat.

If homoserine is a product arising from the conversion of the sulfur of methionine to that of cystine (2-8), the reactions may be reversible and, with an otherwise adequate diet which includes a supply of methyl groups (choline) and sulfur (cystine), the rat might be able to synthesize methionine from homoserine at a rate sufficient for growth. It has recently been demonstrated that in *Neurospora* homoserine furnishes the carbon chain for the synthesis of methionine (9). Thus, in spite of the contraindications obtained in the feeding experiments with the isomers of cystathionine and the *in vitro* studies of their cleavage by liver extract (7, 8), it seemed of interest to determine whether the growing rat was able to carry out the synthesis of methionine from homoserine plus choline and cystine.

A diet devoid of methionine but containing these three compounds was fed to young rats. The results presented herein show that homoserine cannot give rise to methionine under the conditions outlined. Although Teas, Horowitz, and Fling found that in *Neurospora* homoserine is a precursor of threonine as well as of methionine (9), McCoy, Meyer, and Rose (10) several years ago showed that homoserine was not able to replace threonine for the growth of young rats. It thus appears that homoserine has little or no nutritional significance in the diet of the rat.

## EXPERIMENTAL

A litter of young white rats (Sprague-Dawley) was used for the feeding experiment. The basal synthetic diet had the following composition: amino acid mixture (exclusive of the sulfur-containing amino acids) 22.0, sucrose 41.0, Spry 26.0, salt mixture (General Biochemicals, Inc., Salt

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Mixture 2<sup>1</sup>) 4.0, cod liver oil 5.0, and agar 2.0 parts respectively. The amino acid mixture was composed of crystalline amino acids and except for minor alterations was essentially that reported by Rose and Rice (11). The water-soluble vitamins were given as a solution, each rat receiving daily 0.5 cc. of a solution which contained 10  $\gamma$  of thiamine chloride, 10  $\gamma$  of riboflavin, 10  $\gamma$  of nicotinic acid, 10  $\gamma$  of pyridoxine hydrochloride, 50  $\gamma$

TABLE I  
*Results of Growth Experiments on Methionine-Deficient Diet*

Rat No.	Days	Average daily food intake	Average daily weight change	Supplement to basal diet
		gm.	gm.	per cent
1 (♀)	1-8	4.3	-1.6	
	9-14	4.8	+2.0	1.4 methionine
	15-23	4.8	-1.1	2.4 homoserine + 1.2 cystine
	24-29	5.0	+0.7	1.2 homocystine + 1.2 "
2 (♀)	1-8	5.5	-1.8	
	9-14	7.1	+2.2	0.6 methionine + 0.6 cystine
	15-21	7.4	+1.4	0.6 " + 0.6 "
3 (♂)	1-8	5.4	-2.1	
	9-14	4.5	-0.5	0.6 homoserine + 0.6 cystine
	15-20*	4.7	-1.7	0.6 " + 1.2 "
4 (♂)	1-8†	4.1	-3.1	
5 (♂)	1-8	5.4	-2.1	
	9-14	8.0	-0.5	1.2 homoserine + 0.6 cystine
	15-20	4.8	-1.8	1.2 " + 1.2 "
6 (♀)	1-8	4.9	-1.5	
	9-14	4.7	-0.5	2.4 homoserine + 0.6 cystine
	15-21	6.0	-1.0	2.4 " + 1.2 "
7 (♂)	1-8	4.9	-2.3	
	9-14	4.5	-0.5	0.6 cystine
	15-21	5.3	-0.4	1.2 "
8 (♂)	1-8	4.5	-1.6	
	9-14	4.8	-0.5	0.6 cystine
	15-29	5.0	-0.4	1.2 "

\* Animal died after 20 days.

† Animal died after 8 days on the basal diet.

of calcium *d*-pantothenate, and 12.5 mg. of choline chloride. DL-Homoserine, DL-methionine, DL-homocystine, and L-cystine were incorporated in the diets in the amounts shown in Table I and Fig. 1 and an equal weight of sucrose was omitted from the diet. Food and water were allowed *ad libitum*; the food consumption was measured daily and the animals were weighed at intervals.

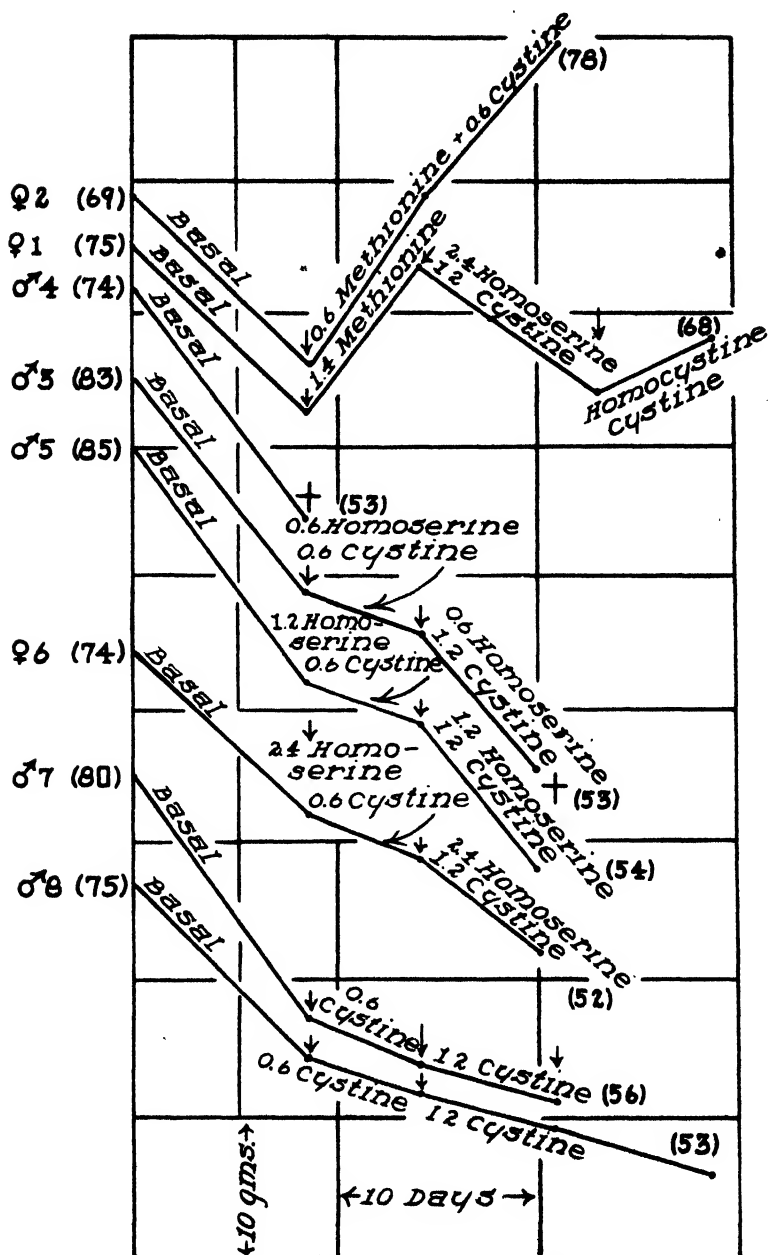


FIG. 1. Growth curves of rats on a methionine-deficient diet. The small vertical arrows indicate a change of diet; the numbers in parentheses indicate the initial and final weights of the animals in gm.

The results of the experiment are tabulated in Table I and are shown graphically in Fig. 1. It is apparent from Fig. 1 that homoserine plus cystine and choline cannot replace methionine in the diet of the rat. The rats receiving homoserine declined faster than the control animals receiving the basal diet plus cystine. An autopsy of Rats 4 and 5 revealed no evidence of gross pathological lesions of either liver or kidney.

#### SUMMARY

It has been found that DL-homoserine in the presence of cystine and choline will not substitute for methionine for the growth of the white rat.

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# THE EFFECTS OF CANAVANINE, ARGININE, AND RELATED COMPOUNDS ON THE GROWTH OF BACTERIA\*

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The physiological properties of canavanine, a naturally occurring, structural analogue of arginine, have not been extensively investigated. Recently, Horowitz and Srb (1) found that canavanine inhibited growth of wild type *Neurospora*, and that the inhibition was competitively alleviated by arginine. Lysine and methionine were also effective in alleviating canavanine inhibition for certain strains of *Neurospora*, though not so effective as arginine. The sensitivity of various *Neurospora* types to inhibition by canavanine varied considerably, and appeared to be genetically determined. These authors review the limited previous work dealing with the physiological response of various organisms to canavanine.

As an extension of this work, the present article describes the effects of canavanine on growth of several lactic acid bacteria. In contrast to wild type *Neurospora*, most of these organisms require arginine for growth. For comparative purposes, two strains of *Escherichia coli* have been used, a parent strain which grows without added arginine, and an arginine-requiring mutant derived from the parent strain by treatment with mustard gas.<sup>1</sup>

## Procedure

**Cultures and Media**—Cultures of *Lactobacillus arabinosus* 17-5, *L. casei*, *L. delbrueckii* 3, *L. fermenti* 36, *Leuconostoc mesenteroides* P-60, and *Streptococcus faecalis* R were carried as stab cultures in yeast extract-glucose-agar. Inocula were grown by transfer from these to a complete liquid

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† Member of the staff of the Daniel Sieff Research Institute, Weizmann Institute of Science, Rehovoth, Palestine.

<sup>1</sup> We are indebted to Dr. J. Lederberg and Dr. E. L. Tatum for cultures of these organisms.



semisynthetic medium similar in composition to that of MacLeod and Snell (2) but with two-thirds of the enzymatically digested casein replaced with acid-hydrolyzed casein. For *L. fermenti* 30  $\gamma$  of extra thiamine were added per 10 cc. After 24 hours incubation, cells were centrifuged and resuspended in 10 cc. of sterile 0.9 per cent sodium chloride solution. This heavy suspension was diluted 1:10 with sterile saline, and 1 drop of this dilute suspension was used to inoculate each experimental culture of 2 cc. For assay, the medium of Henderson and Snell (3), with arginine omitted, was used. For *L. fermenti*, this medium was modified by replacing the sodium citrate with an equal weight of sodium acetate. The initial pH was 6.4.

*Escherichia coli* Y109 (the parent strain) and *Escherichia coli* Y117 (the arginine-requiring mutant) were carried as slant cultures on yeast extract-glucose-agar. For inoculum, a small amount of surface growth from a 24 hour culture was suspended to barely visible turbidity in sterile 0.9 per cent sodium chloride solution, and 1 drop of this suspension was used to inoculate each experimental culture of 2 cc. The basal medium used was that of Tatum and Lederberg (4), which contains inorganic salts, glucose, and asparagine.

Additions of arginine, canavanine, and other compounds were made to 1 cc. of the double strength medium, the cultures were diluted to 2 cc. with distilled water, capped, autoclaved at 15 pounds pressure for 10 minutes, cooled, inoculated, and incubated at 37° for 24 to 38 hours. Cultures were then diluted to 10 cc. with water, and turbidities compared visually or quantitatively in the photoelectric colorimeter.

### Results

*Response of Cultures to Arginine and Related Compounds*—The effects of arginine, citrulline, ornithine, and canavanine on the growth of the various test organisms are compared in Table I. Under the conditions used, each of these organisms except *Escherichia coli* Y109 requires arginine or one of its precursors for growth. *Lactobacillus fermenti* and *Escherichia coli* Y117 can utilize either ornithine or citrulline in place of arginine; *L. arabinosus*,<sup>2</sup> *L. casei*, and *L. delbrueckii* utilize citrulline, but not ornithine, in place of arginine. Finally, organisms such as *Streptococcus faecalis* and *Leuconostoc mesenteroides* utilize neither ornithine nor citrulline, but require preformed

<sup>2</sup> Lyman *et al.* (5) reported that their culture of *Lactobacillus arabinosus* did not require arginine for growth when the medium contained ample vitamin B<sub>6</sub> and the carbon dioxide tension was raised. Under conditions used in this work, no growth occurred without arginine or citrulline during 48 hours of incubation. On prolonged incubation, however, the organism eventually grew without added arginine, and by subculturing from such a culture, a strain of *Lactobacillus arabinosus* was readily derived which grew rapidly without either ornithine, citrulline, or arginine.

arginine. Such data indicate that arginine synthesis in lactic acid bacteria, as in *Neurospora* (6), occurs via the ornithine cycle, and that an apparent requirement for arginine may result from loss of ability to catalyze any one of the several consecutive reactions involved in, or preceding, this cycle.

None of the organisms could effectively utilize canavanine in place of arginine, although high levels of this compound permitted very slight growth of *Leuconostoc mesenteroides*, *Streptococcus faecalis*, and *Lactobacillus fermenti*. The growth-promoting activity here was considerably less than 1 per cent of that of arginine, and may possibly result from the presence of traces of arginine as an impurity in the canavanine. As appears from the

TABLE I

Comparative Effects of Arginine and Related Compounds on Growth\* of Bacteria in Arginine-Free Media

Organism	Additions,† amount per 2 cc.					
	None	DL-Ornithine, 200 $\gamma$	DL-Citrulline, 200 $\gamma$	L-Arginine		L-Canavanine, 1000 $\gamma$
				100 $\gamma$	10 $\gamma$	
<i>L. arabinosus</i> .....	—	—	+++	+++	+	—
" <i>casei</i> .....	—	—	+++	+++	+	—
" <i>delbrueckii</i> .....	—	—	+++	+++	+	—
" <i>fermenti</i> .....	—	+++	+++	+++	+	±
" <i>mesenteroides</i> .....	—	—	—	+++	+	±
<i>S. faecalis</i> .....	—	—	—	+++	+	±
<i>E. coli</i> Y109.....	++	++	++	++	++	—
" " Y117.....	—	++	++	++	+	—

\* —, no growth; ±, barely visible growth; +, easily visible growth; ++, good growth; +++, heavy growth.

† Throughout this table and Tables II and III these compounds were added as DL-ornithine·HCl, DL-citrulline, L-arginine·HCl, and L-canavanine· $\frac{1}{2}$ H<sub>2</sub>SO<sub>4</sub>.

last column in Table I, the growth response of these same three organisms was unaffected by excess canavanine. In separate experiments, concentrations of canavanine sulfate as high as 5 mg. per 2 cc. failed to inhibit the growth response of these organisms to 10  $\gamma$  of arginine; indeed, this response was enhanced by an amount equivalent to the slight growth obtained with the same quantity of canavanine alone. The behavior of these highly resistant organisms stands in direct contrast with that of the other organisms tested. With these, the growth response to arginine was prevented by simultaneous addition of sufficient canavanine. *Escherichia coli* Y109, which grows well without added arginine, was likewise inhibited by canavanine.

Further experiments were conducted with each of the organisms inhibited by addition of canavanine. Similar findings were made in each case; the inhibitory effects of canavanine were alleviated by addition of increased amounts of arginine. Only illustrative data, obtained with organisms of different types, will be given below.

Fig. 1 shows how growth of *Escherichia coli* Y109 is affected by variation of the concentration of arginine and canavanine. This organism grows maximally in the absence of arginine. In the absence of canavanine, additions of arginine do not affect growth. Less than 100  $\gamma$  of canavanine sulfate per 2 cc. of medium completely prevent growth; simultaneous addition of increasing amounts of arginine hydrochloride permits growth even

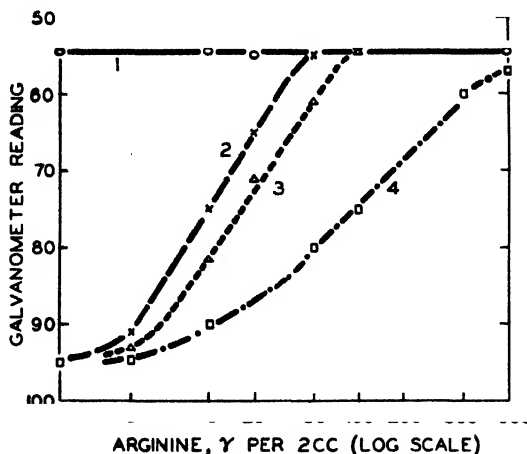


Fig. 1. Effect of concentration of arginine and canavanine on growth of *Escherichia coli* Y109. Curve 1, no canavanine. Curves 2, 3, and 4 obtained with 100, 200, and 1000  $\gamma$  of canavanine sulfate respectively per 2 cc.

in the presence of large amounts of canavanine. The inhibition is competitive in nature; the ratio of canavanine to arginine which permits half maximum growth (galvanometer reading of 75) is 10:1, 12.5:1, and 10:1 respectively, at the three increasing levels of canavanine.

In Fig. 2, the effect of additions of arginine hydrochloride and canavanine sulfate on growth of the arginine-requiring mutant of *Escherichia coli* Y117 is shown. Here again canavanine inhibits growth, and the inhibition is alleviated by additional arginine. In this case, the ratio of canavanine to arginine which permits half maximum growth (galvanometer reading of 60) is approximately 23:1 at the lower concentration of canavanine (1000  $\gamma$  per 2 cc.) and only 8:1 at the higher (2000  $\gamma$  per 2 cc.). Thus, in this instance, arginine is less effective in counteracting canavanine inhibition as

the concentration of the latter is increased. This behavior was noted repeatedly.

*Escherichia coli* Y117 requires about 30  $\gamma$  of arginine per 2 cc. to permit maximum growth (Curve 1, Fig. 2); addition of 2000  $\gamma$  of canavanine does not completely prevent growth at this level of arginine (Curve 3, Fig. 2). The parent strain of *Escherichia coli* Y109 grows maximally without added arginine, since it synthesizes this amino acid. In the absence of arginine, however, less than 100  $\gamma$  of canavanine completely inhibits its growth (Curve 2, Fig. 1). At equivalent levels of growth, therefore, the parent strain, which synthesizes its own arginine, is much more sensitive to the inhibitor. This is to be expected, since the inhibition is dependent upon

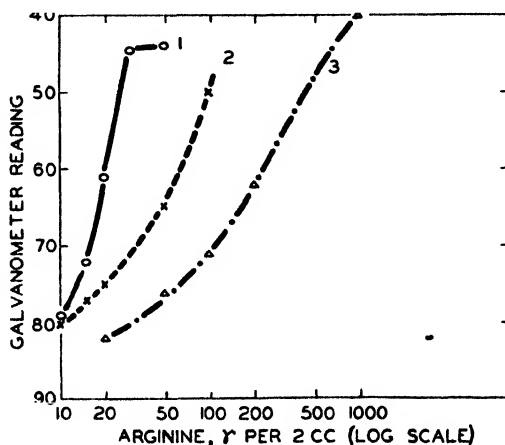


FIG. 2. Effect of concentration of arginine and canavanine on growth of *Escherichia coli* Y117. Curve 1, no canavanine; Curves 2 and 3, obtained with 1000 and 2000  $\gamma$  of canavanine sulfate respectively per 2 cc.

the ratio of the concentrations of arginine and canavanine. In the arginine-synthesizing culture, the arginine is utilized for growth as formed, and presumably never accumulates in excess. Its concentration is always very low, and hence relatively low concentrations of canavanine suffice to prevent growth. When arginine is added to the medium, the sensitivity of the parent and mutant strain to canavanine inhibition is very similar (cf. Curve 4, Fig. 1, and Curve 3, Fig. 2).

Each of the lactic acid bacteria whose growth was inhibited by canavanine (Table I) was investigated in more detail. The findings in each case were similar to those recorded above for the *arginineless* mutant of *Escherichia coli*. In Table II, the arginine concentrations necessary to promote equal growth in the absence and in the presence of various levels

of canavanine are compared. These and many other data not tabulated show that the ratio of canavanine to arginine required to reduce growth to a given level is not constant at various levels of canavanine, but decreases as the canavanine level increases. As the concentration of canavanine is increased, relatively more arginine is required to overcome its inhibitory action. The explanation for these inconstant ratios is not yet known.

From day to day, considerable variation in the inhibition index at any one level of canavanine was seen. Previous experiments (7) with analogues of pantothenic acid showed that increasing the time of incubation decreased the effectiveness of the inhibitor; *i.e.*, increased the inhibition index. This was true also in the present case. A more important factor leading

TABLE II

*Comparative Arginine Concentrations Required by Various Lactic Acid Bacteria for Equal Growth in Presence and Absence of Canavanine\**

Organism	Canavanine sulfate	Arginine hydrochloride	Canavanine to arginine ratio
	$\gamma$ per 2 cc.	$\gamma$ per 2 cc.	
<i>L. casei</i> .....	0	5	
	150	10	15:1
" <i>arabinosus</i> .....	0	8	
	1500	500	3:1
	4000	4000	1:1
" <i>delbrueckii</i> .....	0	10	
	1000	2000	0.5:1
	1000†	200†	5:1†

\* The growth levels selected for comparison are the same with any one organism, but vary from one organism to another from about one-fourth to one-half of maximum growth (24 hours).

† This experiment was conducted in parallel with the one immediately above, but a 3-fold heavier inoculum was used.

to variation was the size of the inoculum. The last two lines of Table II compare the results obtained in parallel assays with *Lactobacillus delbrueckii* when the inoculum was varied over a 3-fold range. With the larger inoculum, the inhibitory properties of canavanine at this concentration have been very considerably decreased.

*Effect of Precursors of Arginine on Canavanine Inhibition*—From Table I it is evident that citrulline and ornithine serve as precursors of arginine. Their effect on the inhibitory properties of canavanine was therefore determined. Results obtained with *Escherichia coli* Y109 are shown in Table III. It is seen by inspection that citrulline and ornithine are approximately equally effective in counteracting canavanine inhibition, but are only about one-twentieth as active in this capacity as arginine. En-

tirely similar data were obtained with *Escherichia coli* Y117, which requires one of these three compounds for growth. For this latter organism, DL-citrulline and DL-ornithine hydrochloride were 0.78 and 0.83 as active, respectively, as L-arginine hydrochloride in promoting growth. In alleviating canavanine inhibition, however, each was less than one-eightieth as effective as arginine.

The effectiveness of canavanine in inhibiting arginine utilization suggested that canaline might show a similar relationship to ornithine utilization. This amino acid<sup>3</sup> was consequently tested (a) for its ability to replace ornithine in promoting growth of *Escherichia coli* Y117 and *Lactobacillus fermenti*, and (b) for its ability to inhibit growth of *E. coli* Y109

TABLE III

*Comparative Effectiveness of Arginine, Citrulline, and Ornithine in Overcoming Inhibition of Escherichia coli Y109 by Canavanine*

	L-Arginine hydrochloride		DL-Citrulline		DL-Ornithine hydrochloride	
		Galvanometer reading*		Galvanometer reading*		Galvanometer reading*
	$\gamma$ per 2 cc.		$\gamma$ per 2 cc.		$\gamma$ per 2 cc.	
No canavanine present	0	55	0	55	0	55
	200	55	500	55	1000	55
200 $\gamma$ canavanine sulfate per 2 cc.	0	95	0	95	0	95
	3	93	20	91	20	88
	10	82	100	85	100	78
	20	71	200	81	200	75
	50	61	500	68	400	67
	100	55			1000	62
	200	56				

\* Arbitrary scale; distilled water = 100, uninoculated medium = 95.

and to prevent the response of *E. coli* Y117 and *Lactobacillus fermenti* to minimum levels (10  $\gamma$  per 2 cc.) of ornithine. It showed no activity in either capacity in amounts up to 1 mg. per 2 cc. Another analogue of ornithine,  $\alpha$ -amino- $\delta$ -hydroxyvaleric acid, was synthesized as described by Sørensen (8), and similarly tested. It showed neither growth-promoting nor growth-inhibiting properties in amounts up to 4 mg. per 2 cc.

#### DISCUSSION

That *Neurospora* synthesizes arginine via reactions of Krebs' ornithine cycle has been shown by the careful investigations of Srb and Horowitz

<sup>3</sup> Because of possible lability of this hydroxylamine derivative to autoclaving with the medium, it was filtered and added aseptically to the previously sterilized medium for these tests.

(6). It is interesting that the same enzymatic deficiencies induced in *Neurospora* by artificial production of mutants are found to occur naturally in various lactic acid bacteria. Thus representatives of these organisms have been found (Table I) whose requirement for arginine can be met by ornithine, citrulline, or arginine; by citrulline and arginine, but not ornithine; and by arginine only. Apparently arginine synthesis in the lactic acid bacteria, too, proceeds via this same series of reactions, illustrating anew the essential similarity of the synthetic mechanisms in different forms of life.

For none of the bacteria studied is canavanine able effectively to replace arginine for growth. Its structural similarity to arginine is reflected, however, in the fact that it inhibits utilization of arginine by many of these organisms. The competitive nature of the inhibition further indicates that inhibition results from the combination of canavanine with cellular constituents normally involved in arginine metabolism. Perhaps in organisms such as *Streptococcus faecalis* which are extremely resistant to inhibition by canavanine the structural features required for such combination are not fully met by canavanine. It is known, for example, that canavanine is a much weaker base than arginine (9). Perhaps, as suggested by Horowitz and Srb (1), such resistant organisms are able to detoxify the inhibitor. In this latter connection, the presence of arginine dihydrolase in *Streptococcus faecalis* and other streptococci of Group D (10), and its possible occurrence in other canavanine-resistant organisms, suggests a mechanism by which the inhibitor might be decomposed. The action of this enzyme on canavanine is not known; the latter is, however, split by a liver enzyme (probably arginase) to yield canaline (11). That such cleavage would in fact detoxify canavanine is shown by the observed inactivity of canaline as an inhibitor.

In contrast to many antimetabolites, such as those of pantothenic acid (7, 12), which are effective inhibitors only for organisms which do not synthesize the corresponding metabolite, canavanine inhibits organisms which synthesize their own arginine as well as those which require preformed arginine. It resembles in this respect the sulfonamides and certain other amino acid inhibitors, such as  $\beta$ -2-thienylalanine (13) and hydroxyaspartic acid (14). It is not necessary, however, to assume a fundamentally different mode of action for these two classes of inhibitors. According to present concepts, the antimetabolite and its corresponding metabolite compete for an enzyme involved in the further transformation of the metabolite. To permit such competition the metabolite must be present in a free and diffusible form. Where an antimetabolite functions against cells which synthesize the metabolite, it may be assumed that the metabolite normally appears in free and diffusible form in the cell preliminary to utilization in the process which the inhibitor affects. In those cases in

which the antimetabolite is ineffective against cells which synthesize the metabolite, this apparently is not true.

Horowitz and Srb (1) found that lysine was also effective in alleviating canavanine inhibition of *Neurospora*. With the organisms used here, lysine was ineffective; mixtures of lysine and arginine were no more effective than would be predicted from their arginine content.

It was pointed out above that, at equivalent levels of growth, *Escherichia coli* Y109 (which synthesizes its own arginine) was much more readily inhibited by canavanine than was *Escherichia coli* Y117, for growth of which arginine (or a precursor) must be supplied. However, the two organisms showed similar sensitivity to canavanine when supplied with equal amounts of arginine. Reasons for such behavior were discussed above. It should be expected, on this basis, that arginine precursors, such as citrulline and ornithine, would be much less effective than arginine in preventing canavanine inhibition, and this was observed in all instances tested. These observations are thus consistent with previous conclusions (e.g. (14)) that precursors of a metabolite are generally less effective than the metabolite itself in preventing inhibition of growth by an antimetabolite. All of our data support the conclusion that inhibition of growth by canavanine results from its interference in the utilization of arginine for various synthetic reactions, presumably the synthesis of cell proteins.

#### SUMMARY

The specificity of the arginine requirement of a number of lactic acid bacteria was examined. Some grew only when arginine was supplied, others with either arginine or citrulline; still others could utilize ornithine, citrulline, or arginine. None of the bacteria tested could effectively use canavanine in place of arginine. Similarly, canaline did not replace ornithine for those organisms which utilize the latter amino acid.

For some, but not all, of the bacteria tested canavanine was an effective growth inhibitor. This was true both for organisms which synthesize arginine, and for those which require this amino acid preformed. For both types of organisms, inhibition by canavanine was competitively alleviated by arginine. In many instances, the ratio of canavanine to arginine at which a given level of growth was observed was not constant, but decreased as the concentration of canavanine was increased. The magnitude of this ratio was markedly dependent upon the size of the inoculum. For organisms which utilized them for growth, citrulline and ornithine showed limited effectiveness in counteracting canavanine inhibition; they were, however, much less effective than arginine. Lysine was ineffective.

Canaline and  $\alpha$ -amino- $\delta$ -hydroxyvaleric acid did not inhibit utilization of ornithine by the organisms tested.

The significance of these various results is discussed briefly.



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# THE DETERMINATION OF ACETONE AND KETONE BODIES

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We have received several inquiries concerning the reproducibility of results with the method described by us for the determination of acetone and ketone bodies (1). Since, in our hands, the results had always been reproducible, an investigation was made to determine the cause of the difficulties encountered by our correspondents.

The method depends on the constancy of the distribution of the 2,4-dinitrophenylhydrazone of acetone between an acid solution and carbon tetrachloride. If equilibrium is not attained, the final results will not be reproducible. Equilibrium is not consistently attained if the extracting solvent, carbon tetrachloride, does not form an emulsion with the acid solution of the hydrazone. To this end, vigorous shaking must be employed in a container whose volume is at least twice that of the solution and solvent. We recommend a glass-stoppered (standard taper 12/40) test-tube of 17 cm. over-all length with an outside diameter of 2 cm. for the extraction.

Since our original description, we have used the Evelyn colorimeter with the small test-tube adapter described by us (2).

The sensitivity of the procedure is increased if measurement is made at the wave-length of the maximum absorption of the 2,4-dinitrophenylhydrazone of acetone in carbon tetrachloride, 350  $m\mu$ ; the absorption at this wave-length is approximately 4 times that at 420  $m\mu$ .

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# THE FATE OF RADIOACTIVE COPPER ADMINISTERED TO THE BOVINE\*

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Recognition of the essentiality of copper for animal life and the availability of suitable chemical analytical methods have led to considerable experimental investigation of copper metabolism. Cunningham (1) surveyed the occurrence of copper in the organs of twelve species of land animals, as well as in plants and foodstuffs. Elvehjem (2), in 1935, presented an extensive review of the biological significance of copper, and Schultze (3) has comprehensively discussed the rôle of this element in blood formation on the basis of experimental evidence available to 1940. The literature on copper in relation to animal health has been reviewed to 1944 by Russell (4). Typical investigations based on chemical analysis include balance and tissue accumulation experiments with rats (5, 6), analysis of blood copper in rabbits and sheep after oral and intravenous administration (7), detailed experiments on the excretion of copper by the rabbit (8), and a study of liver storage in the rat, rabbit, and guinea pig (9).

Emphasis has been placed upon work with sheep by workers in the United Kingdom and Australia, due to the economic importance of a naturally occurring copper deficiency disease in lambs. Sheep, cattle, and goats are known to be affected, and while this condition has not been reported for horses, it has been observed in foals. A naturally occurring copper deficiency in cattle has been observed on the muck soils of Florida (10). In addition to the usual symptoms of anemia, severe diarrhea, cessation of growth, and depigmentation of hair, there was evidence of an abnormal bone metabolism as indicated by rickets-like swellings at the long bones of the calves and rarefaction of bones in the older cattle. It has been reported by English workers (4) that the copper deficiency syndrome in cattle can be caused by an excess of molybdenum in the forage, and, furthermore, that the therapeutic use of copper will protect the animals. Interestingly enough, analysis of the forage grown on Florida muck areas has indicated

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The  $\text{Cu}^{64}$  used in this investigation was supplied by the Clinton Laboratories and obtained on allocation from the United States Atomic Energy Commission.

levels of molybdenum in some cases potentially toxic for livestock; similar levels of molybdenum have been reported also in certain areas of California (11). A research program was designed, therefore, to explore the interrelationships of copper and molybdenum and the possible rôle of these elements in bone metabolism. As a phase of this program, the present paper describes procedures for the use of pile-produced  $\text{Cu}^{64}$  in animal experiments and the behavior of copper administered to the normal bovine.

#### EXPERIMENTAL

The most suitable isotope of copper for tracer studies is  $\text{Cu}^{64}$ , which has a half life of 12.8 hours and emits  $\beta$ -particles (upper limit 0.58 m.e.v.), positrons (upper limit 0.66 m.e.v.), and annihilation  $\gamma$ -rays. In 1942 Schultze and Simmons (12) reported the use of cyclotron-produced  $\text{Cu}^{64}$  in studies on nutritional anemia of rats, and demonstrated the retention of copper in certain organs as well as the entrance of dietary copper into the bone marrow of the rat. Their material was prepared by proton bombardment of nickel to yield about 0.5 millicurie with a specific activity at time of feeding of about 2 millicuries per mg. In 1943 Schubert *et al.* (13) followed  $\text{Cu}^{64}$  after intravenous injection into two dogs; this isotope was prepared by deuteron bombardment of copper to yield about 1.7 millicuries with a specific activity of about 0.05 millicurie per mg. at the time of injection. The irradiation unit as removed from the pile has about 100 millicuries activity, and as it reaches our laboratory some 28 hours later will contain about 20 millicuries with a specific activity of 0.06 millicurie per mg. It may be noted that while cyclotron production of  $\text{Cu}^{64}$  by transmutation will yield higher specific activities, the total activity available is considerably less than from pile production; from the practical standpoint, due to the short half life, this means that cyclotron-produced  $\text{Cu}^{64}$  can be used only with small animals over relatively short time periods and that the experiments must be started soon after conclusion of the bombardment.

A consideration of estimated costs will emphasize the limitations that would be placed on large animals and extended experimentation were it not for the availability of pile-produced  $\text{Cu}^{64}$ ; from the values of Schultze and Simmons (12) with a conservative estimate of \$15 per hour operating expense of the cyclotron, the cost per millicurie at the cyclotron would be about \$150, whereas the corresponding cost at the pile is about \$0.25.

The irradiation unit consists of 0.32 gm. of pure copper wire. The copper is removed from the shipping container, immediately dissolved in a small volume of 8 N nitric acid, and is then neutralized with 2 N sodium hydroxide. A magnetic stirrer is used to provide vigorous agitation during neutralization to avoid precipitation of the hydroxide; the solution is adjusted as close as possible to neutrality without causing precipitation. Measured

amounts are then removed for calibration standards and administration to the animals. Studies have indicated that there is no practical advantage to be gained by purification of the irradiation unit. The half life of each sample as received was determined and found in good agreement with the established value of 12.8 hours. Purification of the copper by extraction from acid solution with dithizone did not change the half life. Perhaps of most significance were our experiments in which the blood plasma was analyzed chemically for copper before and after the administration of the radioactive preparation; the increase in plasma copper as determined chemically agreed fairly well with the values obtained by activity measurements.

It was found convenient to make the radioactivity measurements in solution with the dipping counter as previously described (14). The mica window bell jar type counter was also employed with about equal sensitivity, in which case a given volume of solution was placed in a standard Petri dish directly under the window. Calibration curves were determined daily and the measurements were corrected for decay by calculation to the nearest curve. It was noticed early in the work that with certain radio-copper solutions it was very difficult to get the dipping counter to return to background after a measurement. Later observations indicated that when the pH was above 4, an adsorption to the glass wall of the counter occurred. This indicates that all solutions, both standards and samples, should be kept definitely acid to avoid loss by adsorption during the various steps of the procedure.

Since it was desired to handle at least 200 samples in each experiment, the ordinary method of dry ashing in a muffle furnace was found too time-consuming. A rapid method of pseudo wet ashing, based on solvent extraction of the fat, was developed so that 200 samples could be prepared for measurement within a few hours. In actual practice, the autopsy of a large animal is started at 8 a.m. and the samples are ashed and radioactivity measurements completed within 16 hours. The procedure is as follows: A representative sample, up to 80 gm., is cut into small pieces and placed in a 400 ml. beaker, to which 40 ml. of concentrated nitric acid are added. The tissue is allowed to soak 10 minutes, after which gentle heat is applied. The resulting solution is evaporated to about 15 ml. on a hot-plate and then transferred to a steam bath for continued evaporation to remove the acid. The residue is washed into a separatory funnel with small amounts of warm water. The beaker is rinsed with two 10 ml. portions of isoamyl alcohol which are in turn transferred to the separatory funnel. The separatory funnel is shaken gently and the two solutions separated and made to volume for measurement with the Geiger counter.

Adequate health precautions were employed, since it was necessary to handle 20 millicuries of a  $\gamma$ -ray emitter. Radiation-monitoring instru-

mentation and protective clothing were used. Operations were arranged to be carried out at a distance of about 4 feet from the source, and when it was necessary for personnel to handle the original material at closer range lead shielding was used and the time of exposure minimized. All authoritative suggestions on health protection applicable to this system, from the handling of the original solutions through the measurements and including the final disposition of the animal products, were followed implicitly.

#### RESULTS AND DISCUSSION

*Excretion of Copper*—Yearling bulls, on a normal ration, weighing about 350 pounds, were used for the excretion data presented in Table I. The copper solution was administered by stomach tube or injected into the jugular vein, with the other experimental details as previously described for cobalt (15). About 250 mg. of copper were used for oral administration and about 100 mg. for the injection. The values given represent the ex-

TABLE I

*Excretion during 5 Days of Labeled Copper by Cattle in Percentage of Administered Dose*

Administration	No. of animals	Feces	Urine
Oral .....	1	75	3
Injection.....	3	3	3

cretions during the 5 days following administration, since the decay of the isotope did not allow further observations. The values are probably somewhat lower than the true total excretions, although previous experience has indicated that elimination of most of an unabsorbed element will occur within the first 5 days.

Although only one animal is reported for the excretion of dietary copper, values for the gastrointestinal contents from several other animals sacrificed after 42 hours indicate that the 75 per cent excretion figure is not out of line. This demonstrated that in the bovine the absorption of even soluble copper is quite low. Eden (8) reported that when 50 mg. of copper as the sulfate were administered to a rabbit by stomach tube about 96 per cent of the dose was eliminated in the feces and about 1 per cent in the urine. Houk *et al.* (6) found that rats on diets of varying iron, copper, and cobalt content retained from -6.2 to 3.0 per cent of the dietary copper. Lindow *et al.* (5) reported that rats excreted 98 per cent of supplemental dietary copper in the feces, although on a normal diet the fecal-urinary ratio was about 2. The ratio of fecal to urinary excretion of radioactive copper administered to rats in this laboratory averaged about 25, which agrees with the values in Table I and is of the same order of magnitude as that reported by Eden for rab-

bits. This ratio will depend, of course, upon the total amount and solubility of the ingested copper.

The apparent high retention of intravenously injected copper was quite surprising in view of the previous findings with cobalt (15), where over 60 per cent of the cobalt dose was rapidly eliminated in the urine and over 30 per cent in the feces. However, this behavior confirms a field experiment with cattle, which demonstrated that injected copper exhibited a much longer period of therapeutic efficiency than did orally administered copper. Eden and Green (7) reported that of 3 mg. of copper injected into the ear vein of a rabbit about 17 per cent appeared in the urine within 48 hours, and the remaining 82 per cent in the feces over a period of 4 weeks, with most of the fecal excretion occurring during the first 2 weeks.

TABLE II  
*Partition of Labeled Copper in Blood of Cattle*

Administration	Time	Labeled copper dosage in blood	Labeled copper in blood contributed by plasma
		<i>per cent</i>	<i>per cent</i>
Intravenous injection .....	5 min.	36	63
“ “ .....	1 hr.	36	31
“ “ .....	3 hrs.	19	9
“ “ .....	6 “	13	12
“ “ .....	19 “	9	13
“ “ .....	26 “	7	14
“ “ .....	44 “	5	33
Oral .....	19 “	0.15	39
“ .....	44 “	0.29	50

*Fate of Copper in Blood Stream*—Table II presents data showing the per cent of the labeled copper dosage found in the blood and the proportion of the labeled copper in the blood which was contributed by the plasma, at varying times after administration. The animals described earlier were used, and the total blood was calculated as 7.7 per cent of the body weight. The blood samples were taken from the jugular vein opposite that used for the injection.

The injected copper was rapidly removed from the blood stream by capillary passage through the tissues, as only 36 per cent of the dose was found in the blood after 5 minutes; the removal of copper from the blood was slower thereafter. It is generally considered (7), at least for sheep and cattle, that normal blood copper is equally distributed between the plasma and cells. The values in Table II lead to the following picture in regard to the behavior of intravenously injected copper. Immediately after



injection two processes occur, removal of copper from the plasma by deposition in the tissues, and absorption of copper by the red blood cells. The first process must occur initially at a much faster rate than the second, since at 5 minutes about 64 per cent of the dosage was removed from the blood and yet the plasma still contained a greater proportion of the labeled copper than did the cells. Between 5 minutes and 1 hour, there was practically no removal of copper from the blood; however, the proportion in the plasma decreased, which may be taken to indicate a continued accumulation by the cells during this period. Between 1 and 3 hours there was further removal of copper from the blood with the indication that most, if not all, of the copper was being removed by the tissues from the plasma. From then on, the attainment of equilibrium between the red blood cell and plasma copper proceeded at a faster rate than removal of copper from the blood. However, even after 44 hours normal distribution was not attained. The data show that such large deviations from the normal distribution do not occur when the copper is absorbed from the tract. The accumulation of copper in the red blood cells is not simply a heavy metal adsorption; otherwise the same behavior would be expected with cobalt, and in similar previous work (15) no cobalt was detected in the cells. These findings agree in general with those of Eden and Green (7), who used chemical analytical methods with rabbits and sheep, and with those of Schubert *et al.* (13), who employed radioactive copper with dogs.

*Tissue Distribution of Copper in Bovine*—Table III shows the tissue distribution of labeled copper injected into a 2 year-old bull and orally administered to a young bull calf and another 2 year-old bull. The procedure for the slaughter of the animals and the handling of the tissues has been described earlier (15).

Considering the distribution of the ingested copper, it is evident that, although the retention by the animal is low, the copper which is absorbed is generally distributed and does reach the tissues involved in hematopoiesis. The tissues showing a high concentration were, in decreasing order, the liver, kidney, gastrointestinal tract, adrenals, thymus, and gallbladder and bile; those of medium concentration include the pancreas, red bone marrow, intestinal lymph, blood, spleen, heart, lung, and reproductive organs; very low concentrations of copper were found in the white bone marrow, muscle, hide, bladder, ligament, cartilage, bone, eye, and nerve tissue. It must be remembered that in some cases negative results will be due in part to the small sample available. It is obvious that the liver, which shows the highest concentration, serves as the chief storage organ. The kidney invariably has the next highest concentration, and in the adult bovine the ratio of liver to kidney accumulation averages about 5; in tissue distribution studies with rats sacrificed after 17 to 65 hours, we have found the concentration of labeled copper in the kidney to exceed that in the liver,

TABLE III

*Tissue Distribution of Labeled Copper Administered to Cattle*

Mode of administration..... Age and weight of animal..... Actual dosage, mg. Cu..... Sacrificed after, hrs.....	Oral 1½ mos., 85 lbs. 146 42		Oral 18 mos., 450 lbs. 256 42		Jugular injection 18 mos., 460 lbs. 160 19	
	γ per 100 gm. fresh weight per 100 mg. dosage	Per cent of dose in whole tissue	γ per 100 gm. fresh weight per 100 mg. dosage	Per cent of dose in whole tissue	γ per 100 gm. fresh weight per 100 mg. dosage	Per cent of dose in whole tissue
Pituitary	*	0	*		35	0.00059
Thyroid	*	0	*		17	0.0046
Thymus	32	0.032	3	0.0067	19	0.024
Adrenals	48	0.0015	3	0.00045	28	0.0033
Reproductive organs	5	0.0017	1	0.0058	8	0.056
Cerebrum	*	0	*		1	0.0020
Cerebellum	33	0.008	*		2	0.0016
Eye	*	0	*		6	0.0042
Intestinal lymph glands	13		3		29	
Heart	8	0.019	1	0.012	11	0.088
Blood	10	0.29	2	0.25	60	9.4
Aorta	*	0	3	0.0019	23	0.013
Lung	8	0.037	1	0.022	66	0.96
Trachea	7	0.0031	*		10	0.021
Kidney	71	0.10	9	0.045	150	0.72
Bladder	4	0.0013	1	0.007	20	0.014
“ urine	69	0.0066	17	0.040	29	0.010
Tongue	6	0.011	2	0.015	10	0.068
Esophagus	3	0.0012	2	0.0059	14	0.029
Fundus abomasum, mucosa	42	0.042	6	0.032	27	0.15
“ “ muscular	13		3		10	
Pyloric “ mucosa	44		11		33	
“ “ muscular	9		3		12	
Abomasum contents	100	0.12	39	0.57	2	0.023
Reticulum	17	0.011	4	0.025	28	0.15
Omasum	100	0.075	3	0.046	13	0.16
“ contents	690	0.38	61	2.0	*	
Rumen	13	0.033	2	0.055	33	1.2
“ and reticulum contents	310	7.8	40	11.0	*	
Duodenum, mucosa	*	0.38	10	0.097	51	0.28
“ muscular	10		*		20	
Jejunum, mucosa	31		4		34	
“ muscular	16		3		15	
Ileum, mucosa	32		4		20	
“ muscular	14				18	
Small intestine contents	50	0.21	70	2.2	6	0.15
Large “ mucosa	45	0.10	8	0.12	61	1.8
“ “ muscular	13		4		12	
“ “ contents	310	0.67	750	29.1	18	0.6

TABLE III—*Concluded*

Mode of administration.....	Oral 1½ mos., 85 lbs. 146		Oral 18 mos., 450 lbs. 256		Jugular injection 18 mos., 460 lbs. 160	
Age and weight of animal.....	146		256		160	
Actual dosage, mg. Cu.....	42		42		19	
Sacrificed after, wks.....						
	γ per 100 gm. fresh weight per 100 mg. dosage	Per cent of dose in whole tissue	γ per 100 gm. fresh weight per 100 mg. dosage	Per cent of dose in whole tissue	γ per 100 gm. fresh weight per 100 mg. dosage	Per cent of dose in whole tissue
Pancreas	17	0.0074	2	0.0024	87	0.21
Spleen	9	0.012	3	0.015	43	0.20
Liver	1090	7.0	41	1.0	1130	32.6
Gallbladder and bile	97	0.0056	2	0.0036	190	0.1
Tenderloin muscle	6		1			
Gastrocnemius muscle	*		*		4	
Ligament	*		1		11	
Cartilage	*		*		7	
Bone	*		*		*	
Red bone marrow (ribs)	15		2		51	
White bone marrow (long bones)	9		*		21	
Teeth	*				*	
Hide	5		1		9	

γ represents micrograms of Cu.

\* No radioactivity detected in sample.

which points to a species difference and confirms the results of Schultz and Simmons (12). With rabbits, the liver-kidney ratio of labeled copper has ranged from 13 to 30.

Chemical analysis has shown that the livers of very young animals are especially high in copper and that a calf is born with a copper concentration in the liver of 4 to 8 times that in the liver of the adult; the data in Table III confirm this selectivity and indicate that it persists at least through the first 1½ months of life. In comparing the values from the young and the adult animals, it is better to use the percentage of dose figures, since the concentrations are affected by the body weight. It is noted that except for the liver the percentages of dose found in the various tissues are in close agreement; this indicates that the liver of the calf actually exhibits a greater selective accumulation of the absorbed copper.

The injection values in Table III were obtained from an animal of approximately the same weight as the adult receiving the copper orally, and, therefore, in this case both the concentration and percentage dose values are comparable. In general, the injection tissue values are about 10 times the corresponding ingestion values, which again reflects the poor absorption of the orally administered copper. The proportional distribution of the injected copper was similar to that of the ingested copper.

Since it is recognized that the gastrointestinal tract has an important hematopoietic function, particular attention has been paid to the accumulation of copper in the true stomach and intestinal mucosa. It may be noted from Table III that in all cases the mucosal was greater than the muscular concentration; negative values for the duodenum were due in part to the small sample available. The ratio of mucosal to muscular concentration averaged 2.7 for the ingested and 2.8 for the injected copper. When the copper was injected, the accumulation in the mucosa probably occurred via the general circulation, since it has been shown that relatively small amounts of copper reach the intestinal contents during the 24 hours following injection. In the case of the true stomach particularly, it would seem unlikely that the mucosal accumulation of the injected copper could have come from the contents. It is considered, then, that the gastrointestinal mucosa does concentrate copper from the general circulation, but an estimate of the extent of secretion into the lumen must await bile and pancreatic fistula studies.

To date, fifteen cattle have been used for studies with copper as reported here; there has been general agreement, and for brevity only results from representative individuals have been presented. The normal behavior of copper in the bovine, as herein described, is considered a basis for the study of deficiency and abnormal conditions.

#### SUMMARY

Procedures are described for the use of pile-produced  $\text{Cu}^{64}$  in studies with large as well as small animals. The results are in general agreement with such findings as are available in the literature.

Only a small proportion of orally administered copper is absorbed by the bovine; yet that which is absorbed reaches practically every tissue. Intravenously injected copper is generally distributed and highly retained at least during the 1st week after administration.

Immediately following intravenous injection, copper is rapidly removed from the plasma by the tissues and is more slowly accumulated by the red blood cells. The red blood cell copper is more tenaciously retained than the plasma copper as far as removal by the tissues is concerned, although equilibrium between the plasma and red blood cell copper is eventually approached.

During the first  $1\frac{1}{2}$  months of life the liver of the calf exhibits a greater selective accumulation of absorbed copper than is observed with the older animal.

Tissue accumulations of orally administered and intravenously injected copper are presented and discussed.

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# THE UPTAKE OF GLUCOSE AND THE SYNTHESIS OF GLYCOGEN BY THE ISOLATED DIAPHRAGM OF NORMAL AND PITUITECTOMIZED RATS

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Clinically, the antagonistic actions of the hormones of the anterior pituitary and of the islands of Langerhans of the pancreas have been well documented. Davidoff and Cushing (1) reported that the incidence of diabetes in acromegalic patients was 12 per cent in their series and 35 per cent in Borchard's series as compared with 0.5 per cent in the general population (2). Basophilic adenomas of the pituitary are also accompanied by hyperglycemia and insulin resistance (3, 4). Conversely, hypoglycemia and extreme insulin sensitivity are cardinal features of the hypopituitary state (4).

Physiological experiments by Houssay (5) and Geiling (6) have shown antagonistic action of pituitary extract and insulin. Houssay discovered that the removal of the anterior pituitary gland suppressed the diabetes of the pancreatectomized animal and that the subsequent injection of the anterior pituitary extract caused hyperglycemia to reappear. Houssay (5) and Geiling *et al.* (6) have demonstrated that pituitectomized dogs are very sensitive to the hypoglycemic effects of insulin.

By *in vitro* studies with isolated diaphragm muscle of the rat, Gemmill (7, 8) has demonstrated that insulin increased glucose disappearance from the medium and glycogen synthesis in the isolated diaphragm. Stadie and Zapp (9) and Corkill and Nelson (10) have confirmed this. Corkill and Nelson also reported that the antecedent injection of anterior pituitary extract intraperitoneally in rats prevented the insulin effect on the glucose utilization and glycogen synthesis.

Cori (11), studying the effect of purified muscle hexokinase upon the phosphorylation of glucose, reported that insulin had no *direct* accelerating effect on this reaction. However, whereas anterior pituitary extract inhibited the hexokinase reaction, insulin abolished this anterior pituitary inhibition. He concluded that he could not demonstrate a direct action of insulin on the hexokinase reaction but that insulin did counteract the inhibiting action of anterior pituitary. This action of insulin failed to explain

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the extreme insulin sensitivity of the hypophysectomized animal. Cori, therefore, suggested that insulin might also have a direct stimulatory effect on some other enzyme reaction.

Since the first step in glucose utilization, the hexokinase reaction, is irreversible, Krahl and Cori (12) stated that it should be the rate-determining step for glucose uptake by the diaphragm. In order to extend their studies of the action of insulin on the hexokinase reaction to intact muscle, they utilized Gemmill's technique in their study of the diaphragms of normal, diabetic, and adrenalectomized rats.

Stadie (9), discussing the implications of Cori's data, stated "if the action of insulin is *solely* to release the inhibitory action of anterior pituitary extract on the hexokinase reaction, no effect of insulin *in vitro* should be found when diaphragms from hypophysectomized rats are used." Stimulated by previous work in Stadie's laboratory and by this statement, one of us (M. P.) repeated Gemmill's experiments with normal rats and rats 4 to 5 days after hypophysectomy. Both the glucose disappearance from the medium and the glycogen synthesis by the isolated diaphragm were measured.

### *Methods and Materials*

**Animals**—All the rats were females of the Hisaw strain, weighing between 125 and 175 gm. when killed. The animals were fed Purina dog chow *ad libitum* in the first series. In the second experiment, the rats were tube-fed 10 ml. of a fluid food mixture per day for 3 to 4 days before they were killed. The fluid mixture contained 0.6 gm. of Cellu flour, 0.02 gm. of Phillips and Hart Salts 4,<sup>1</sup> 0.5 gm. of dried yeast, 0.8 gm. of casein, 1.8 gm. of starch, 0.75 gm. of dextrin, 0.7 gm. of sucrose, 1 ml. of Mazola oil, and water to a final volume of 10 ml.

The normal rats were fasted for 24 hours before the experiment and the pituitectomized rats were fasted for 18 hours.

All pituitectomies were performed by one of us (R. O. G.) using the parapharyngeal approach. The completeness of the pituitectomy was confirmed by postmortem examination of the sella with the aid of a dissecting microscope.

**Experimental Methods**—The rats were stunned by a blow on the head and then decapitated. The diaphragms were removed according to the description of Stadie and Zapp (9). One-third of each hemidiaphragm was used to determine the initial glycogen content of the diaphragm. The rest of the hemidiaphragms were placed in Warburg vessels which had been kept at 4°. The large compartment of each vessel contained 2 ml. of Krebs' Ringer-

<sup>1</sup> Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **133**, 460 (1941).

phosphate solution (pH 7.4) with either 0.3 or 0.1 per cent glucose. 0.2 ml. of 1 N NaOH was placed in the center well to absorb the respiratory  $\text{CO}_2$ . To remove the phenol, used as a preservative in commercial crystalline insulin solutions, the insulin was subjected to isoelectric precipitation. 0.001 N NaOH was added to the commercial insulin, drop by drop, until a dense cloud appeared. The tube was then spun at 4000 revolutions per second for 15 minutes. The clear supernatant was decanted and tested for residual insulin by the addition of more NaOH and the 0.001 N HCl. If no further cloud appeared, the supernatant was discarded. The precipitate was dissolved in distilled water by the addition of a few drops of NaOH to bring the pH to 7.0. The concentrated insulin solution contained 60 units per ml. Just prior to the experiment, the concentrated solution was diluted 1:100 with distilled water. 0.033 ml. of this dilute solution was added to the main compartment of alternate Warburg flasks. The final concentration of the insulin in the medium was thus 0.01 unit per ml. These small quantities of insulin were used because the concentrations were closer to the normal concentration of serum and because previous experiments had demonstrated that the insulin effects with this low concentration were similar to those produced by 0.5 to 1 unit of insulin per ml.

The Warburg vessels, containing the diaphragms in the medium, were gassed for 3 minutes with 100 per cent oxygen and then placed in a Warburg water bath at 38°. The flasks were shaken at a rate of 68 times per minute. After an incubation period of 2 hours, the vessels were removed from the bath. The diaphragms were immediately removed and placed in 1 ml. of 30 per cent KOH solution. 1 ml. of the residual medium was pipetted into flasks containing  $\text{ZnSO}_4$  for a Somogyi precipitation prior to glucose analysis.

Glycogen was analyzed by the method of Good, Kramer, and Somogyi (13). The protein in the media, at the end of the experiment, was precipitated by the method of Somogyi (14). The glucose was determined by a modification of Benedict's method (15).

### Results

The amount of glucose disappearing from the medium and the amount of glycogen in the diaphragms are expressed as mg. of glucose per 100 mg. of wet diaphragm per 2 hours of incubation.

The glucose concentration of the medium in the first series of experiments was 0.3 per cent and in the second it was 0.1 per cent.

*Diaphragms from Normal Rats in 0.3 Per Cent Glucose Medium*—The mean amount of glucose utilized without insulin was 0.64 mg. per cent and 0.99 mg. per cent with insulin; the insulin effect was 0.35 mg. per cent (55 per cent increment). The glycogenesis without insulin was 0.30 mg. per



cent and 0.60 mg. per cent with insulin; the insulin effect was 0.30 mg. per cent (100 per cent increment) (Table I).

*Diaphragms from Pituitectomized Rats in 0.3 Per Cent Glucose Medium*—The mean amount of glucose utilized without insulin was 1.06 mg. per cent and 1.66 mg. per cent with insulin; the insulin effect was 0.60 mg. per cent (57 per cent increment). The mean glycogenesis without insulin was 0.30

TABLE I

*Glucose Uptake and Glycogenesis by Diaphragms of Normal Rats*

Each line gives the data of the diaphragm of one rat. The glucose concentration of the medium was 0.3 per cent. The vessels were incubated at 38° for 2 hours. The glucose uptake and glycogenesis are expressed as mg. of glucose per 100 mg. of wet weight of diaphragm.

Rat No.	Glucose uptake			Initial glycogen content	Glycogenesis		
	Without insulin	With insulin	Insulin effect		Without insulin	With insulin	Insulin effect
1				0.13	0.28	0.55	0.27
2				0.06	0.32	0.76	0.44
3				0.17	0.19	0.52	0.33
4				0.14	0.30	0.54	0.24
5				0.13	0.19	0.68	0.49
6	0.94	1.29	0.35				
7	0.50	0.83	0.33				
8	0.63	1.02	0.39				
9	0.71	1.24	0.53	0.26	0.23	0.68	0.45
10	0.34	0.83	0.49	0.16	0.26	0.47	0.21
11	0.56	0.77	0.21	0.16	0.30	0.51	0.21
12	0.89	1.07	0.18	0.04	0.41	0.55	0.14
13	0.58	0.83	0.25	0.07	0.39	0.51	0.12
14				0.10	0.51	0.84	0.33
Mean.....	0.64	0.99	0.35 (55%)	0.13	0.30	0.60	0.30 (100%)
S.E.M.....			±0.043				±0.04
t.....			8.1				7.5

mg. per cent and 0.51 mg. per cent with insulin; the insulin effect was 0.21 mg. per cent (70 per cent increment) (Table II).

The pituitectomized rats in this series were fed *ad libitum* postoperatively. On the 4th and 5th day postoperatively, they were killed. All of the eleven rats ate poorly and lost some weight. Three of these were almost moribund on the day of the experiment.

The sella turcica was examined post mortem only in Rats 7 to 11. About one-quarter of the pituitary (posterior lobe) was found in the sella of Rat 8. The other four sellae contained no pituitary tissue.

By tube feeding both the control and the pituitectomized animals and by decreasing the glucose content of the medium in the Warburg vessels from 6 to 2 mg., two undesirable experimental features of the preceding experiments were eliminated. These were (1) the difference in nutritional state between the pituitectomized and control rats, and (2) the determination of the glucose utilization represented a small difference between two large numbers, since only 0.5 to 1 mg. of the 6 mg. in the medium was

TABLE II

*Glucose Uptake and Glycogenesis by Diaphragms of Pituitectomized Rats*

Each line gives the data of the diaphragm of one rat. The glucose concentration of the medium was 0.3 per cent. The vessels were incubated at 38° for 2 hours. The glucose uptake and glycogenesis are expressed as mg. of glucose per 100 mg. of wet weight of diaphragm.

Rat No.	Glucose uptake			Initial glycogen content	Glycogenesis		
	Without insulin	With insulin	Insulin effect		Without insulin	With insulin	Insulin effect
1*	1.06	2.44	1.38	0.26	0.24	0.49	0.25
2*	1.13	2.14	1.01	0.18	0.17	0.58	0.41
3	0.89	1.19	0.30	0.11	0.26	0.48	0.22
4	1.67	1.83	0.16	0.08	0.31	0.32	0.01
5	1.07	1.83	0.76	0.18	0.17	0.51	0.34
6*	1.42	2.20	0.78	0.10	0.38	0.46	0.08
7	0.74	1.19	0.45	0.03	0.35	0.52	0.17
8	1.11	1.33	0.22	0.11	0.30	0.49	0.19
9	1.09	1.35	0.26	0.10	0.63	0.70	0.07
10	0.60	1.25	0.65	0.03	0.32	0.65	0.33
11	0.84	1.50	0.66	0.03	0.23	0.43	0.20
Mean . . . . .	1.06	1.66	0.60 (57%)	0.11	0.30	0.51	0.21 (70%)
S.E.M. . . . .			±0.11				±0.04
t . . . . .			5.5				5.4
t† . . . . .	1.00	1.43	0.43 (43%)	0.07	0.32	0.51	0.19 (59%)

\* Almost moribund.

† Excluding the three moribund animals.

utilized. These changes were considered desirable in order to prevent the wide variation of insulin effects on glucose uptake noted in Table II.

*Diaphragms from Normal Rats in 0.1 per cent Glucose Medium*—Six animals were tube-fed for 3 to 4 days before the experiment. The last tube feeding was given 24 hours before the experiment. The mean amount of glucose utilized without insulin was 0.53 mg. per cent and 0.85 mg. per cent with insulin; the insulin effect was 0.32 mg. per cent (60 per cent increment). The initial glycogen content was 0.18 mg. per cent; this was higher than

TABLE III

*Glucose Uptake and Glycogenesis by Diaphragms of Normal Rats*

Each line gives the data of the diaphragm of one rat. The glucose concentration of the medium was 0.1 per cent. The vessels were incubated at 38° for 2 hours. The glucose uptake and glycogenesis are expressed as mg. of glucose per 100 mg. of wet weight of diaphragm.

Rat No.	Glucose uptake			Initial glycogen content	Glycogenesis		
	Without insulin	With insulin	Insulin effect		Without insulin	With insulin	Insulin effect
1	0.54	0.93	0.39	0.17	0.18	0.46	0.28
2	0.39	0.82	0.43	0.18	0.09	0.46	0.37
3	0.49	0.66	0.17	0.19	0.19	0.33	0.14
4	0.50	0.72	0.22	0.21	0.13	0.29	0.16
5	0.65	0.93	0.28	0.08	0.28	0.47	0.19
6	0.58	1.02	0.44	0.25	0.07	0.30	0.23
Mean . . . . .	0.53	0.85	0.32 (60%)	0.18	0.16	0.39	0.23 (144%)
S.E.M. . . . .			±0.046				±0.035
t . . . . .			6.9				6.6

TABLE IV

*Glucose Uptake and Glycogenesis by Diaphragms of Pituitectomized Rats*

Each line gives the data of the diaphragm of one rat. The glucose concentration of the medium was 0.1 per cent. The vessels were incubated at 38° for 2 hours. The glucose uptake and the glycogenesis are expressed as mg. of glucose per 100 mg. of wet weight of diaphragm.

Rat No.	Glucose uptake			Initial glycogen content	Glycogenesis		
	Without insulin	With insulin	Insulin effect		Without insulin	With insulin	Insulin effect
1	0.42	0.60	0.18	0.08	0.15	0.23	0.08
2	0.35	0.58	0.23	0.03	0.21	0.29	0.08
3	0.23	0.53	0.30	0.10	0.13	0.32	0.19
4	0.41	0.73	0.32	0.07	0.15	0.26	0.11
5	0.27	0.52	0.25	0.13	0.09	0.23	0.14
6	0.33	0.70	0.37	0.27	0.12	0.28	0.16
7	0.30	0.73	0.43				
8	0.46	0.94	0.48	0.16	0.08	0.29	0.21
9	0.38	0.79	0.41	0.14	0.16	0.39	0.23
10	0.47	0.89	0.42	0.04	0.16	0.39	0.23
Mean . . . . .	0.36	0.70	0.34 (94%)	0.11	0.14	0.30	0.16 (114%)
S.E.M. . . . .			±0.03				±0.02
t . . . . .			11				8

that of other series. Although these high initial glycogen contents might have been due to the forced feeding, the initial contents in hypophysectomized animals similarly fed were lower. The glycogenesis without insulin was 0.16 mg. per cent and 0.39 mg. per cent with insulin; the insulin effect was 0.23 mg. per cent (144 per cent increment) (Table III).

*Diaphragms from Pituitectomized Rats in 0.1 Per Cent Glucose Medium*—Ten animals were tube-fed for 3 to 4 days after the operation. Thus their food intake was identical with that of the control rats. The animals were killed 16 to 18 hours after the last tube feeding. These animals were in a good state of nutrition and activity when they were killed. The sella of every animal was examined post mortem and no pituitary tissue was found.

The mean amount of glucose utilized without insulin was 0.36 mg. per cent and 0.70 mg. per cent with insulin; the insulin effect was 0.34 mg. per cent (94 per cent increment). The initial glycogen was 0.11 mg. per cent. Glycogenesis without insulin was 0.14 mg. per cent and 0.30 mg. per cent with insulin; the insulin effect was 0.18 mg. per cent (114 per cent increment). The insulin effect on glycogen synthesis was slightly but not significantly lower in the two series of pituitectomized rats than in the normal control series. The difference between the mean insulin effect on glycogen synthesis was  $0.09 \pm 0.05$  (standard error of difference of two means) and  $0.07 \pm 0.04$  in these two series (Table IV).

#### DISCUSSION

The insulin effect on glucose utilization of the diaphragm of the pituitectomized animals was as great as the effect on the diaphragms of the normal control rats. The mean insulin effects on the glucose utilization of our four series, as well as the insulin effects of similar series of normal rats gathered from the literature, are presented in Table V. Although the insulin effects vary considerably in the different series, those of our pituitectomized tissue are within the range of the insulin effects noted with normal tissue. The insulin effect reported by Gemmill seems to be greater than the effect reported by Stadie, Corkill, and in the present paper. The reason for this is not apparent.

The observation that the insulin effect on glucose utilization of pituitectomized tissue is not less than that of normal tissue agrees with the clinical and physiological observations that insulin is very effective in increasing glucose utilization in the hypopituitary state.

Our experiments with pituitectomized rats do not support the assumption that the action of insulin is solely upon the hexokinase system. Four possibilities of reconciling Cori's data with our observations can be considered. First, pituitary hormones may still be exerting their effect 4 to 5 days after pituitectomy. This seems very unlikely, since there is no

evidence of positive action of pituitary hormones at such intervals after pituitectomy. On the contrary, there is abundant evidence that the hormonal effect ceases by 48 hours after pituitectomy. After pituitectomy, growth ceases almost immediately (17), the secretion of milk stops within 24 hours (18, 19), and there is loss of adrenal cortical volume at 48 hours

TABLE V

*Comparison of Glucose Uptake and Glycogenesis in These Series with That of Other Reported Series*

Each line represents the mean of the data of each series. Glucose uptake and glycogenesis are expressed as mg. of glucose per 100 mg. of wet diaphragm for the entire duration of the experiment. The insulin effects are expressed both as mg. per 100 mg. of wet diaphragm and as per cent increase.

Author	Concentration of glucose in medium	Period of incubation	Fed or fasted	Glucose utilization			Glycogenesis				
				Without insulin	With insulin	Insulin effect	Without insulin	With insulin	Insulin effect		
Normal											
	<i>per cent</i>	<i>hrs.</i>		<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>per cent</i>
This series	0.3	2	Fasted	0.64	0.99	0.35	55	0.30	0.60	0.30	100
Stadie (16)	0.3	2	"	0.60	1.13	0.53	88				
" (9)	0.3	2	"					0.44	1.04	0.60	136
Gemmell (8)	0.3	3	"	0.59	1.46	0.87	147			0.50	
" (8)	0.2	3	"	0.54	1.03	0.49	91			0.45	
Corkill (10)	0.2	3	Fed	0.47	0.72	0.26	55	0.31	0.58	0.27	87
This series	0.1	2	Fasted	0.53	0.85	0.32	60	0.16	0.39	0.23	144
Krahl (12)	0.1	2	Fed, fasted	0.38	0.50	0.12	32				
Stadie (16)	0.1	2	Fasted	0.43	0.75	0.32	74				
" (9)	0.1	2	"					0.35	0.67	0.32	91
Pituitectomized											
This series	0.3	2	Fasted	1.06	1.66	0.60	57	0.30	0.51	0.21	70
" " *				1.00	1.43	0.43	43	0.32	0.51	0.19	59
" "	0.1	2	"	0.36	0.70	0.34	94	0.14	0.30	0.16	114

\* Excluding three moribund animals.

(20). The interruption of all gonadal functions except that of the corpus luteum in certain pregnant animals is evident in 48 hours or less (21, 22). Maintenance of the luteal function in the pituitectomized pregnant animals is believed to be due to substances originating from the placenta. Second, insulin may act directly on the hexokinase system in the intact cell but not on purified hexokinase. Third, insulin may speed up glucose

utilization by an enzyme system other than hexokinase in the isolated diaphragm. Since the diaphragm, which contracts constantly throughout life, differs from most skeletal musculature in function, it is possible that its enzyme system differs from that of other striated muscle. Gemmill (7) failed to find the *in vitro* insulin effect on glucose utilization of frog sartorius muscle which he had found in the rat diaphragm. It has been demonstrated that cardiac musculature, which also contracts regularly throughout life, differs from skeletal musculature in its ability to form glycogen from lactate (23). Fourth, substances other than pituitary hormones may inhibit the hexokinase reaction. Broh-Kahn and Mirsky (24) have recently reported an inhibition of hexokinase by splenic extracts similar to the reported pituitary inhibition; insulin overcame either of these inhibitions *in vitro*. Thus insulin might be able to increase phosphorylation of glucose even in tissue of pituitectomized animals, if non-pituitary hexokinase inhibitors were present.

Glucose utilization was greatest in the tissue of the moribund fasted pituitectomized rats (Table II). Six normal rats were fasted for 4 days; the glucose utilization of the diaphragms of these rats was greater than that of the controls which were fasted 24 hours. This suggests that prolonged inanition may have been a factor in increasing the glucose utilization of the pituitectomized rats which were fed *ad libitum*.

#### SUMMARY

The effect of insulin upon glucose utilization *in vitro* is as great in the diaphragm of the pituitectomized rat as in the diaphragm of the normal. Insulin can therefore directly increase the diaphragm's utilization of glucose, even in the absence of the inhibitory effect of anterior pituitary hormones.

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# BIOCHEMICAL STUDIES ON HOG TESTICULAR EXTRACT

## I. ISOLATION AND IDENTIFICATION OF 5-PREGNEN-3( $\beta$ )-OL-20-ONE\*

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### PLATE 1

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Interesting theories concerning the biochemical interrelationship of steroid hormones have been presented by many investigators. Selye (1) has summarized these views in a scheme for a possible route of biogenesis of the different types of hormonal compounds. Unequivocal proof for such a scheme would seem to be quite difficult. However, one approach to this problem might involve the examination of specific tissues for the constituent steroid compounds which have hormone activity or which are judged, by their structure, to be closely related to the active compounds. One must assume that the coexistence of substances is indicative of metabolic interrelationship. The common embryonic origin of gonadal and suprarenal cortical tissues suggests that it might be possible to find compounds related to sex hormones and adrenal cortical hormones in a common location. With this in mind we have applied a standard procedure for the isolation of adrenal cortical hormones (2) to the investigation of hog testes extract.

During the early stages of the separation various fractions have been screened for four types of biological activity; adrenal cortical, androgenic, estrogenic, and progestational. This has resulted in the detection of a significant amount of estrogenic activity, as measured by the Kahnt-Doisy assay procedure (3). Although the presence of such activity in hog testes has been reported previously (4), it was not further characterized. At the present time, we are engaged in the identification of this estrogenic material. Our results will be reported in a subsequent paper.

Although adrenal cortical activity has not been detected by a muscle fatigue test (5) or a survival-growth assay (6), a considerable amount of unidentified material was obtained in fractions corresponding to the biologically active cortin fraction from adrenal glands. It is known that, of the twenty-five or so steroid compounds which have been isolated from

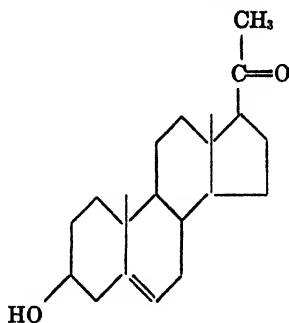
\* A preliminary report of this work was presented at the meeting of the Division of Biological Chemistry of the American Chemical Society in New York, New York, September 15, 1947.



the adrenal cortex, only six have appreciable biological activity by the above methods of assay (7). Thus it is quite possible that this fraction from hog testes may be found to contain compounds such as the so called inactive adrenal steroids.

Investigation of several fractions by means of a standard assay procedure for progesterone (8) has failed to detect this type of hormone activity. Likewise, the use of a modified test, patterned after that of Korenchevsky *et al.* (9), involving the measurement of seminal vesicle and prostate gland development, has not detected androgenic activity. After this work had been completed, a paper (10) came to our attention which reported the isolation of androgenic activity from hog testes. The level of this activity was extremely low, about 450  $\gamma$  of testosterone equivalent per kilo of tissue, and was detected by a chick comb-growth assay (11). Our failure to confirm these findings might be accounted for by our use of a different isolation scheme and a different method of bioassay. Dingemanse *et al.* (12) and Koch (13) have previously discussed the dissimilarity in the response obtained with these two types of assay.

In his scheme for the hypothetical biosynthesis of steroid hormones, Selye (1) has assigned a key position to 5-pregnen-3( $\beta$ )-ol-20-one, commonly



called pregnenolone. Thus any effort to establish an interrelationship of steroid compounds by means of an isolation technique should quite naturally include an attempt at the detection of this substance. Although pregnenolone is readily available as a synthetic compound, having been first prepared in 1934 by Butenandt, Westphal, and Cobler (14), its isolation from a natural source had been reported by only one laboratory prior to the initiation of our investigation. In their work, Ruzicka and Prelog (4) obtained a crystalline mixture of pregnenolone and allopregnanolone, from which they were unable to separate free pregnenolone. Only after acetylation and subsequent chromatography over aluminum oxide were they able to isolate pregnenolone acetate. In our laboratory it has been possible to isolate free pregnenolone as such by means of a somewhat

different isolation scheme, followed by chromatography over magnesium silicate-celite mixtures. Since pregnenolone has no distinguishing biological or chemical properties, it is a difficult substance to follow through any extensive isolation scheme. We have partially overcome this difficulty by applying the very useful tool of infra-red spectroscopy. After the absorption spectrum for synthetic pregnenolone was established, each fraction from the chromatograms of the ketonic material from the tissue extract was examined in the infra-red and compared to the standard curve. By this means it was possible to observe the progressive increase in pregnenolone content of the fractions, which led ultimately to the isolation of the pure compound. The identity of the natural product with synthetic pregnenolone was then established by the standard techniques of melting point, mixed melting point, optical rotation, and the preparation of a chemical derivative. In addition, the Debye-Scherrer x-ray powder patterns and the infra-red absorption spectra in Nujol mulls proved the crystal structure identity as well as the chemical identity of the natural and synthetic compounds.

Two other crystalline compounds were isolated in the course of this investigation. One of these, which melted at  $256^{\circ}$  and possessed an  $[\alpha]_D$  value of  $-15^{\circ}$ , was recovered from a chromatogram of mixed ketones. This compound was in a band adjacent to one from which pregnenolone was isolated. The identity of this substance has not yet been established. The other crystalline compound was characterized as free cholesterol. Although the presence of this sterol in the extract was not unexpected, its point of appearance in the isolation scheme was thought to be unusual.

#### EXPERIMENTAL

The processing involved in this investigation has been divided for convenience of discussion into five stages.

*Stage A. Initial Processing of Hog Testes*—The starting material for this study consisted of 470 kilos of hog testes. These glands had been collected and frozen at the packing-house, after which they were packed in dry ice and shipped to our laboratory.<sup>1</sup> Since it was desired to study the steroid constituents in their natural state in the tissue, every precaution was taken that no transformations should occur previous to or during the isolation study. The frozen glands were ground into 95 per cent acetone (containing 5 per cent water) and processed as shown in Table I. The subsequent processing of fractions IX and VI from Table I is recorded in Stages B and C, respectively.

*Stage B. Processing of Ethylene Dichloride Extract IX*—The details of

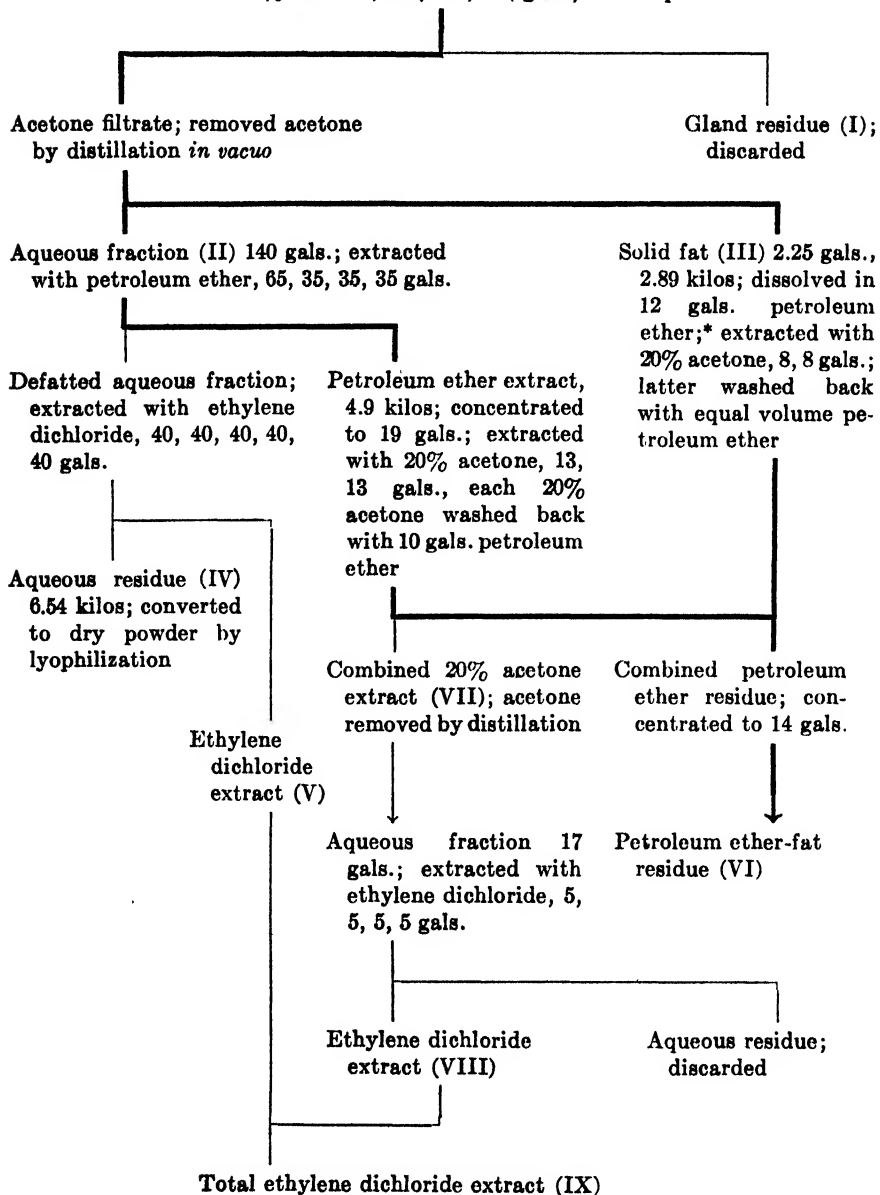
<sup>1</sup> These glands were made available to us by the Reliable Packing Company, Chicago, Illinois.

TABLE I

*Stage A. Initial Processing of Hog Testes*

470 kilos frozen hog testes

Extracted with 95% acetone, 320, 110, 110 gals.; 48 hrs. per extraction



\* Skellysolve B.

TABLE II

*Stage B. Processing of Ethylene Dichloride Extract IX*

Total ethylene dichloride extract IX

Solute transferred to 4 liters S. D. 3A alcohol;\* cooled to 5°

Alcohol mother liquor 373.3 gm.; adjusted alcohol concentration to 70% to make volume 8.57 liters; extracted with 3 liters petroleum ether; latter washed back with 70% alcohol, 2, 2 liters	Crystalline precipitate (X) 136.5 gm.; treated with Darco; crystallized from 3A alcohol, absolute methanol, absolute acetone
	Free cholesterol (XI)
70% alcohol residue; concentrated to 2 liters; adjusted alcohol concentration to 50%; extracted with petroleum ether: 3, 3, 2 liters; latter washed back with 50% alcohol, 2, 2 liters	Petroleum ether extract (XII) 294.8 gm.
50% alcohol residue 44.8 gm.; diluted to 30% alcohol to make volume 6.9 liters; extracted with petroleum ether: 3, 3 liters; latter washed back with 30% alcohol: 1.5, 1.5 liters	Petroleum ether extract (XIII) 22.8 gm.
30% alcohol residue (XV) (42.56 gm.); concentrated to aqueous residue, 4 liters; extracted with ethyl acetate: 2, 2, 2, 2 liters	Petroleum ether extract (XIV) 2.24 gm.
Ethyl acetate extract (XVI) 14.4 gm.; extracted with two 0.1 volumes each 1% $\text{Na}_2\text{CO}_3$ , 0.5 N HCl, 2% $\text{NaHCO}_3$ ; followed by three 0.1 volumes distilled water; each aqueous extract washed through same 4 liters ethyl acetate	Aqueous residue; discarded
Neutral ethyl acetate fraction (XVII) 7.28 gm.	Aqueous washes; discarded

\* S. D. 3A alcohol (90% ethanol, 5% methanol, 5% water) used for all subsequent aqueous alcohol extractions.

the further handling of this fraction are presented in Table II. After the crude crystalline material (X), which gave a strongly positive Liebermann-Burchard test, had been decolorized with Darco and then crystallized from 95 per cent alcohol, a fraction was obtained which melted unsharply at 126–138° and possessed an  $[\alpha]_D^{20}$  value of  $-26^\circ$  ( $c = 1.9$  in chloroform). After recrystallization of this material once from absolute methanol and three times from absolute acetone, a crystalline fraction was obtained which melted sharply and was identified as free cholesterol (XI) by the following properties.



Calculated. C 83.90, H 11.99; m.p. 148°;  $[\alpha]_D^{25} - 39^\circ$

Found.<sup>2</sup> “ 83.88, “ 11.85; “ 144–145°;  $[\alpha]_D^{25} - 38^\circ$ ; ( $c = 1.80$  in chloroform)

The occurrence of this amount of free cholesterol, 290 mg. per kilo of tissue, was not expected at this point in the isolation scheme.

Fraction XVII would be expected to contain the compounds related to the adrenal cortex steroids. In spite of the fact that no adrenal hormone activity was detected, further investigation of this material may yield certain of the so called inactive adrenal compounds. It is interesting to note that this fraction was found to contain 726,000 I.U. of estrogenic activity (equivalent to 72.6 mg. of estrone), as measured<sup>4</sup> by the Kahnt-Doisy assay (3). An investigation of this estrogenic material is under way at the present time.

*Stage C. Processing of Petroleum Ether-Fat Residue VI*—The route by means of which fraction VI evolved in this study may be traced by the heavy lines in Table I. The subsequent handling of this material is outlined in Table III. Ultimately fraction XXIX was produced. No adrenal cortical, androgenic, or progesterone-like activity was detected in this fraction. However, the Kahnt-Doisy assay indicated a total of 2,460,000 I.U. of estrogen activity (equivalent to 246 mg. of estrone).

*Stage D. Preparation of Neutral Ketone Fraction*—An aliquot of fraction XXIX amounting to 5 gm. in 578 ml. of ethyl acetate was used for exploratory studies. It was extracted with four 50 ml. portions of 2 per cent aqueous sodium hydroxide, and each alkaline extract was washed back through the same 100 ml. of ethyl acetate. The alkali washes were combined, acidified, and extracted with ethyl acetate. After being washed with distilled water, the ethyl acetate solution of dilute alkali-soluble

<sup>2</sup> The chemical analyses were carried out by Mr. Harold Emerson and Mr. William Struck of our microanalytical laboratory.

<sup>3</sup> All melting points were taken on a Fisher-Johns block, which had been standardized against a Bureau of Standards thermometer.

<sup>4</sup> We are indebted to Mr. Stanley C. Lyster of our laboratory for the estrogenic assay data which are reported in this paper.

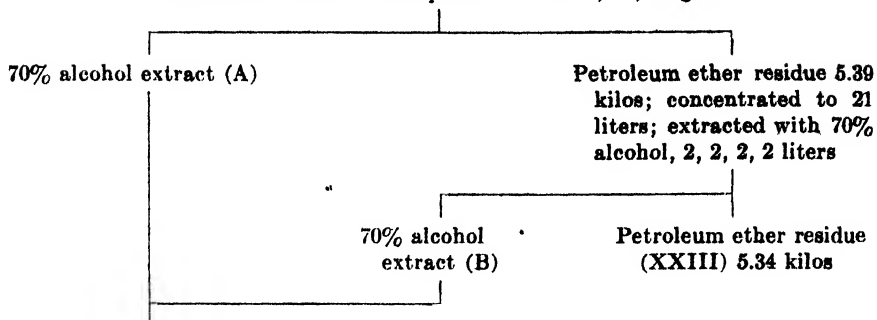
TABLE III

Stage C. Processing of Petroleum Ether-Fat Residue VI

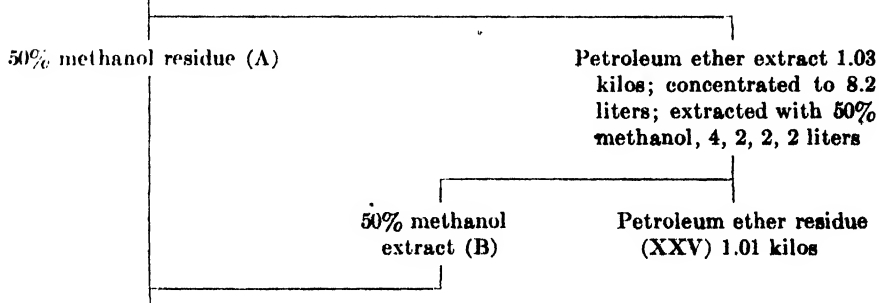
Petroleum ether-fat residue VI, 14 gals.

Extracted with 70% alcohol, 7, 7, 7, 7, 7 gals.

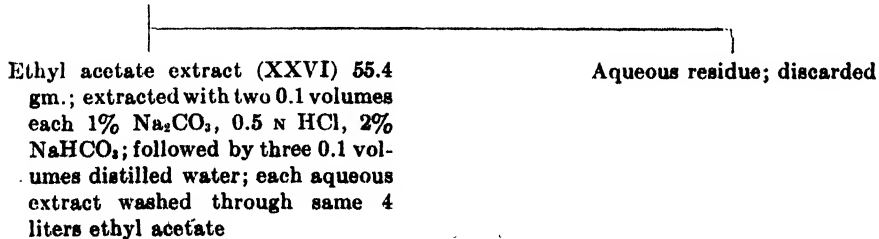
Latter washed back with petroleum ether, 17, 17 gals.



Total 70% alcohol extract (XXII)  
1.08 kilos; alcohol removed by distillation *in vacuo*; aqueous residue adjusted to 50% methanol; 12 gals.; extracted with petroleum ether, 3, 3, 3, 3 gals.



Total 50% methanol extract (XXIV)  
275.5 gm.; concentrated to aqueous residue, 4 liters; extracted with ethyl acetate, 2, 2, 2, 2, 2 liters



Neutral ethyl acetate fraction (XXIX) 14.36 gm.

Aqueous washes; discarded

material (XXXI) contained 840 mg. The ethyl acetate solution of material not extracted with dilute alkali (XXX) contained 4.34 gm.

The latter fraction was subjected to an exhaustive separation with Girard's Reagent T, as described by Ruzicka and Prelog (4). This separation yielded three successive ketone fractions of 588, 87, and 29 mg. and a "non-ketone 3" fraction of 2.97 gm. (XXXIII). A very striking Liebermann-Burchard reaction was obtained when 8 mg. of the latter fraction were dissolved in 2 ml. of chloroform and treated with 0.5 ml. of acetic anhydride and 0.25 ml. of concentrated sulfuric acid. The upper layer became yellow-green, the interphase was red-brown, and the lower layer was tan. A small, bright blue band ascended from the interphase into the upper layer.

The three ketone fractions were pooled to give fraction XXXII of 722 mg., which was subjected to a fourth treatment with Girard's Reagent T. This yielded "non-ketones 4" of 231 mg., fraction XXXV; and "ketones 4" of 396 mg., fraction XXXIV.

The remaining 9 gm. aliquot of fraction XXIX was subjected to a similar separation. The dilute alkali-soluble fraction was pooled with fraction XXXI to give a combined dilute alkali-soluble fraction (XL) weighing 1.61 gm. The exhaustive Girard separation on the dilute alkali-insoluble material gave a non-ketonic fraction which was pooled with comparable material from the first separation to yield a combined non-ketone fraction (XLI) of 8.98 gm. The ketone fraction (XLII) from this separation weighed 850 mg.

Fractions XL and XLI were examined by the Kahnt-Doisy assay and found to contain estrogenic activity equivalent to 8 and 135 mg. of estrone, respectively. However, the chemical properties of this estrogenic material, as exhibited in the preceding separations, would seem to indicate that it is neither estriol nor estrone. At the present time we are engaged in a further investigation of the estrogenic activity in these two fractions and in fraction XVII.

*Stage E. Chromatographic Separation of Ketone Fractions XXXIV and XLII*—These two ketone fractions from Stage D served as the starting material for the isolation of 5-pregnen-3( $\beta$ )-ol-20-one, commonly called pregnenolone. Two types of adsorbents were used in the chromatographic separations; magnesium silicate-Celite mixtures and aluminum oxide.<sup>5</sup> Much sharper separations were obtained with the former. The technique of exhaustive elution was employed, and the resulting column bands were

<sup>5</sup> Magnesium silicate No. 34 was obtained from the Philadelphia Quartz Company, Berkeley South Gate, California. Celite is an analytical filter aid made by Johns-Manville. The aluminum oxide, 80 to 200 mesh, was purchased from the Fisher Scientific Company.

determined on a weight basis. Each band was characterized by optical rotation and infra-red spectroscopy.<sup>6</sup> The infra-red spectrum of each column band was compared to that obtained for synthetic pregnenolone.<sup>7</sup> In this manner the location of pregnenolone could be conveniently determined during the extensive purification scheme on samples of 0.5 to 2 mg. Non-crystalline and semicrystalline fractions were examined as smears on the sodium chloride blocks and could be recovered by washing with dry ether or dry chloroform. Crystalline fractions were examined as Nujol mulls or as chloroform solutions. Column bands which were practically pure pregnenolone, as evaluated by the infra-red spectroscopist, were pooled for subsequent crystallization. Those which contained pregnenolone absorption together with other types were pooled for re-chromatography.

Fraction XXXIV, containing 396 mg. of mixed ketones, was dissolved in 12 ml. of benzene<sup>8</sup> and added to a column (15 × 2 cm.) of a mixture of 5 gm. of magnesium silicate plus 5 gm. of Celite, which had been thoroughly washed with each of the several solvents to be used in the chromatogram. The results with this column are presented in Table IV. Particular attention is called to Band C, which consisted of crystalline material eluted from the column by a mixture of benzene-ether (10:1). It weighed 16.9 mg. and had an  $[\alpha]_D^{20}$  value of +31°. Examination by infra-red indicated a high percentage of pregnenolone. The long needles were washed with a small amount of cold, absolute ether, leaving 5.6 mg. of material melting at 187–188° and showing no depression when melted with synthetic pregnenolone. To this fraction, contained in a tared, 15 ml. graduated centrifuge tube, were added 12.5 mg. of hydroxylamine hydrochloride, 0.08 ml. of distilled water, and 0.05 ml. of 10 per cent aqueous sodium hydroxide. Absolute ethanol was added to make the volume to 0.5 ml. This mixture was kept at its boiling point for 10 minutes. It became quite cloudy when placed in an ice bath, and considerable crystalline material separated upon standing. The mother liquor was withdrawn after centrifugation, and the crystals were dried to a constant weight of 1.2 mg. They melted at 214–216°. No depression was observed when this material was melted with an oxime prepared under identical conditions from 5 mg. of synthetic pregnenolone.

<sup>6</sup> The authors are greatly indebted to Dr. Foil A. Miller and Mrs. Agatha R. Johnson, Division of Physical Chemistry, University of Illinois, Urbana, for their able handling of the infra-red spectroscopy which is reported in this paper.

<sup>7</sup> The sample of synthetic pregnenolone was kindly furnished to us by Dr. Percy L. Julian and Dr. J. Wayne Cole of the Research Laboratories, The Glidden Company, Chicago, Illinois.

<sup>8</sup> All solvents used in this investigation were dried and then distilled in an all-Pyrex apparatus and stored in glass-stoppered Pyrex bottles.



Band D, which was eluted from this column with benzene-ether (4:1 and 2:1), possessed an  $[\alpha]_D^{25}$  value of  $+18^\circ$  and weighed 43.3 mg. Crystallization of this fraction from absolute methanol yielded two crops of crystals weighing 7.4 and 2.2 mg. A third crop of 3.5 mg. was obtained from isopropanol, leaving a mother liquor of 30.2 mg. The latter now had an  $[\alpha]_D^{25}$  value of  $+23^\circ$ , and exhibited a high concentration of pregnenolone in

TABLE IV

*Chromatogram of Neutral Ketones from Hog Testes Extract*

Starting material, 396 mg. of fraction XXXIV; adsorbent, 5 gm. of magnesium silicate plus 5 gm. of Celite; column, diameter 2 cm., length 15 cm.; volume of eluate per fraction, 20 ml.; total recovery, 360 mg. or 91 per cent.

Developer	Fraction No.	Column bands				
		Band	Weight	$[\alpha]_D^{25}$	Description	Infra-red index*
			mg.	degrees		
Petroleum ether-benzene (1:1)	1-8	A	12.3	$+14 \pm 2^\dagger$	Dark yellow oil	—
Benzene	9-11	B	9.5	$+33 \pm 2$	Light " "	++
Benzene-ether (40:1)	12					
" (20:1)	13-14					
" (10:1)	15-17	C	16.9	$+31 \pm 4$	Colorless crystals	+++
" (10:1)	18-22					
" (4:1)	23					
" (2:1)	24-28	D	43.3	$+18 \pm 5$	Crystals + oil	++
" (2:3)	29-30					
Ether	31-32	E	35.5	$+19 \pm 1$	Light yellow oil	—
Ether-methanol (40:1)	33-37	F	90.8	$+9 \pm 2$	Orange oil	—
" (20:1)	38-41	G	28.8	$+15 \pm 2$	Yellow "	—
" (2:1)	42-43	H	54.1	$+10 \pm 2$	Oil	—
Methanol	44-51	I	68.8	$+12 \pm 4$	Red-brown oil	—

\*  $+5 = 100$  per cent pregnenolone.

† The  $\pm$  errors were calculated from the standard equation for  $[\alpha]_D$  by substituting for "average observed rotation" the mean deviation from the mean of the actual observed rotations for each sample.

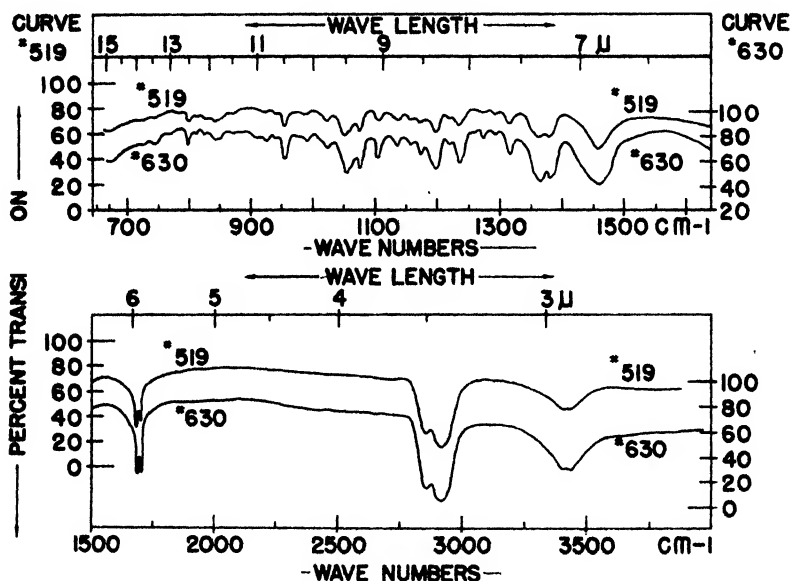
its infra-red spectrum. It was added to similar fractions in a subsequent chromatogram. The three crops of crystals obtained previously were now pooled, and the total fraction was found to have an  $[\alpha]_D^{25}$  value of  $-15^\circ$  ( $c = 0.34$  in absolute methanol). Subsequent recrystallization from absolute methanol yielded 4.4 mg. of colorless platelets, melting at  $256-258^\circ$  and having the chemical composition of C 83.80, H 10.62. As yet, this material has not been identified.

Fraction XLII, containing 850 mg. of mixed ketones, was dissolved in 20 ml. of benzene and treated with 20 ml. of petroleum ether, causing the separation of a resinous precipitate. The latter was dissolved and reprecipitated from 8 ml. of petroleum ether-benzene (1:1). The combined petroleum ether-benzene solution (1:1) contained 757 mg. This fraction was added to a column (12 × 2 cm.) containing 40 gm. of aluminum oxide, which had been washed with all the solvents to be used in the chromatogram. The solvent mixtures used for developing the column were identical with those used in the first chromatogram. The first band, eluted with petroleum ether-benzene (1:1), contained 259.2 mg. and possessed an  $[\alpha]_D^{20}$  value of +29°. Although the physical characteristics of this material were somewhat different from the crude fraction which was added to the column, extensive background absorption prevented definitive characterization by infra-red. However, there was a strong possibility that this fraction consisted of less polar compounds which had not been retained on the column, perhaps due to the use of an inadequate adsorbent. It is seen that 497.8 mg. of the starting material were held on the adsorbent. Subsequent development led to a typical chromatogram. A band, which was eluted with benzene-ether (4:1) and weighed 10.7 mg., was found to have an  $[\alpha]_D^{20}$  value of +29° and exhibited a high concentration of pregnenolone in the infra-red.

A 252 mg. aliquot of the first band from the above column was dissolved in 16 ml. of petroleum ether-benzene (1:1) and chromatographed over a mixture of 5 gm. of magnesium silicate plus 5 gm. of Celite, as in the first column. 47.4 mg. of crystalline material appeared in a band eluted with benzene-ether (10:1), having an  $[\alpha]_D^{20}$  value of +21 ± 1°. This fraction, while contained in a tared, 15 ml. graduated centrifuge tube, was dissolved in 0.3 ml. of boiling absolute methanol and allowed to crystallize. The colorless needles were washed with three 0.1 ml. portions of cold ether to give 7.2 mg. of crystals melting at 187–188° and showing no depression when melted with synthetic pregnenolone. The mother liquors from the above crystallization, as well as several column bands from preceding chromatograms which exhibited infra-red evidence for pregnenolone, were pooled and chromatographed repeatedly over mixtures of magnesium silicate-Celite until all possible pregnenolone had been isolated.

A total of 20.9 mg. of crystalline material was accumulated which had approximately the correct melting point for pregnenolone. Subsequent crystallization of this fraction from absolute methanol and finally from absolute acetone yielded 13.4 mg. of crystals melting at 186.5–187.5° and showing no depression when melted with synthetic pregnenolone. This material was found to have an  $[\alpha]_D^{20}$  value of +35 ± 4° ( $c = 0.25$  in absolute methanol). Further proof of the identity of this natural product with

synthetic pregnenolone was found by comparisons of the Debye-Scherrer x-ray powder patterns<sup>9</sup> (Fig. 1) as well as the infra-red absorption spectra (Text-Fig. 1).<sup>10</sup> The infra-red curves were obtained on samples of the crystalline compounds suspended as mulls in Nujol. Any slight variation between the two curves can probably be explained on the basis of the mull of the natural product being slightly less dense than that of the synthetic sample. Thus, a few of the weaker bands of the natural product are not seen. There are certain points in these curves, such as the twin bands in the "ketone region" at 1685 and 1700  $\text{cm}^{-1}$ , which are probably related to



TEXT-FIG. 1. Infra-red spectra of 5-pregnen-3( $\beta$ )-ol-20-one. Curve 519, natural product; Curve 630, synthetic product. Perkin-Elmer infra-red spectrometer, No. 12-B.

the crystal structure of the compounds. These modifications and others are discussed in a study of the polymorphism of pregnenolone by means of x-ray and infra-red spectroscopy, which is the subject of a separate report. In addition it was found that infra-red spectra of chloroform solutions of the natural product and synthetic pregnenolone were absolutely superim-

<sup>9</sup> The authors wish to express their thanks to Dr. George Pish of our Physics Department for the preparation and interpretation of the x-ray data which are

<sup>10</sup> In preparing these plates, the drafting was done by Mr. John Hoffman of our Mechanical Development Department, and the photography was handled by Mr. Norman A. Drake of our Physics Department.

posable. Thus it can be said that the results with x-ray and infra-red, as reported here, prove not only the chemical identity but also the crystal identity of the natural product and synthetic pregnenolone.

#### SUMMARY

Hog testicular extract has been subjected to an extensive isolation procedure in order to investigate the interrelationship of steroid hormonal compounds. Although no adrenal cortical hormone activity was detected, a fraction was obtained which could contain the so called inactive adrenal steroids. A significant amount of estrogenic activity was found. Pregnenolone (5-pregnen-3( $\beta$ )-ol-20-one) was isolated and identified.

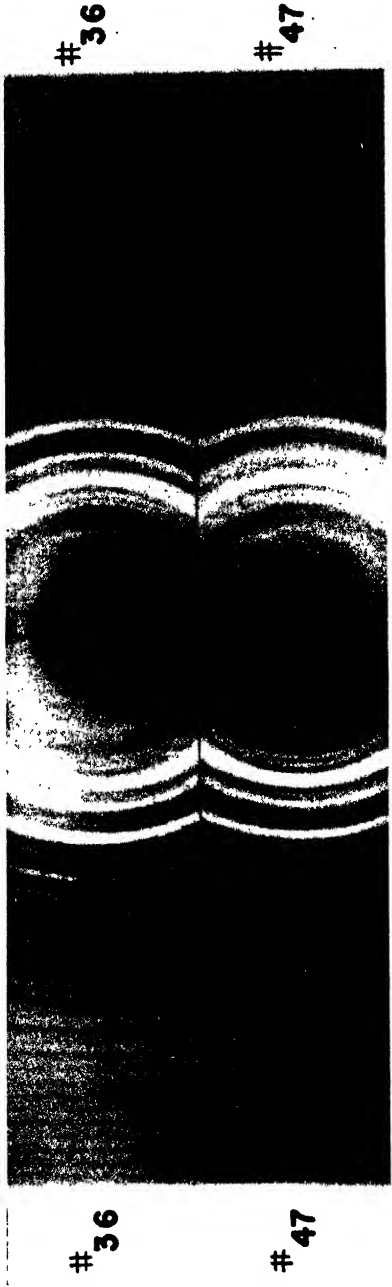
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#### EXPLANATION OF PLATE I

FIG. 1. X-ray powder patterns of 5-pregnen-3( $\beta$ )-ol-20-one. Sample 36, natural product; Sample 47, synthetic product. Picker x-ray diffraction unit, Debye-Scherrer camera, copper radiation, nickel filter, KVP-48, MA-12.





(Haines, Johnson, Goodwin, and Kuizenga: Testicular extract. I)



# THE UPTAKE OF GLUCOSE BY THE ISOLATED DIAPHRAGM OF NORMAL AND HYPOPHYSECTOMIZED RATS\*

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It was reported (1) that the hexokinase reaction, glucose + adenosine triphosphate  $\rightarrow$  glucose-6-phosphate + adenosine diphosphate, in extracts of muscle from diabetic rats could be inhibited by adrenal cortical extract, while the hexokinase activity in extracts of normal muscle was inhibited by a combination of anterior pituitary and adrenal cortical extracts. The inhibition was in both cases relieved by insulin.

In view of these findings, it became of interest to determine the extent to which the hexokinase reaction in muscle is controlled by insulin, adrenal cortical, and pituitary secretions. To this end, observations on the rate of glucose uptake by rat diaphragm were begun. This rate should be a measure of hexokinase activity in the muscle, since the hexokinase reaction is presumably the initial step in glucose utilization and is irreversible.

In the initial series of experiments (2) it was found that the rate of glucose uptake by diaphragm from diabetic rats was depressed. This inhibition, appearing when insulin was lacking, could be counteracted, in part, by addition of insulin to the diaphragm *in vitro* or by adrenalectomy. Villee, Sinex, and Solomon (3), using  $C^{14}$ -labeled glucose, have reported that glucose use, glycogen deposition, and carbon dioxide production were decreased in diaphragms from diabetic rats and increased in diaphragms from adrenalectomized rats.

The present experiments were carried out to determine whether hexokinase activity was increased in diaphragms from rats lacking pituitary secretions and whether insulin would have any effect on the reaction under these circumstances.

## Methods and Materials

**Animals**—Young male rats from Anheuser-Busch, Inc. (Strain AB), and Sprague-Dawley, Inc. (Strain SD), were used. They were fed a diet of commercial dog biscuit described previously (2).

**Hypophysectomized Rats**—The entire pituitary was removed by the para-

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pharyngeal approach.<sup>1</sup> Hypophysectomized rats were maintained on the stock diet and water *ad libitum*, and were weighed twice weekly. About 10 per cent of the hypophysectomized animals were rejected because of continued weight gain, persistent testes, or gross pituitary residues.

At necropsy, an examination for hypophyseal remnants was made. The region around the wound, the attached stalk, and the overlying brain were fixed. Serial sagittal sections, which included the entire wound and the stalk, were examined microscopically.<sup>2</sup> In an effort to detect residual anterior pituitary cells in the wound, two types of specific staining were tried: a modified Mallory's trichrome stain following Bouin fixation (4), and an azocarmine method following Zenker fixation.<sup>3</sup> No true anterior pituitary basophils or acidophils were found. There were, however, occasional small groups of glandular cells around the tip of the stalk or elsewhere in the wound. These cells were considered to be pars tuberalis on the basis of size of cells, the size, shape, and location of the nucleus, and the cytoplasmic staining. These residues were small; there subsequently proved to be no correlation between the presence or absence of such residues and the glucose uptake of the diaphragm of the animal in question.

The adrenals were weighed and then fixed for histological examination. In confirmation of Deane and Greep (5) it was found that adrenals from hypophysectomized animals exhibited specific regression of the fascicular layer of the cortex. At 8 to 12 days after operation the thickness of the fascicular zone was about one-half to two-thirds that of the controls; by 70 days after operation the atrophy had progressed to the point where the fascicular zone was so thin as to be barely distinguishable.

*Procedure for Handling Diaphragms*—The diaphragm was removed as soon as possible after death and immersed immediately in ice-cold Krebs-Henseleit solution, previously equilibrated with 95 per cent oxygen-5 per cent carbon dioxide, which contained the same concentration of glucose as that subsequently to be used for measurement of glucose uptake. Each hemidiaphragm was cut transversely, yielding four pieces in all.

Each piece was blotted and transferred to the chilled medium contained in the small cylindrical vessels previously used (2) or in 10 ml. Erlenmeyer flasks. The vessels containing medium and tissue were next attached to Warburg manometers by means of a rubber sleeve and equilibrated with the gas phase (95 per cent oxygen-5 per cent carbon dioxide). A channel to allow escape of gas between the glass of the manometer tip and the rubber

<sup>1</sup> The hypophysectomies were performed by the staff of the Hormone Assay Laboratories, Chicago.

<sup>2</sup> The authors are indebted to Mr. Joseph Albrecht for technical assistance in the preparation of slides.

<sup>3</sup> Finerty, J. T., unpublished work.

sleeve can be maintained by means of a piece of wire which is removed after the equilibration.

The vessels were shaken in a bath at 37° for the desired period, then removed, stoppered, and chilled in ice water. Samples for determination of residual glucose were removed and analyzed as previously described (2). Finally, the pieces of diaphragm were blotted and weighed on a torsion balance.

Preliminary experiments showed that the rate of glucose utilization by normal diaphragm decreased rapidly during 2 hours incubation. These initial experiments also indicated that the magnitude of the stimulation of glucose uptake produced by 1 unit per ml. of insulin was greater at a 5 to 6 cm. amplitude of shaking than at a 2 cm. amplitude, presumably because of the better access of insulin to individual muscle fibers under conditions of greater agitation. It is now believed that this is the explanation for the fact that the percentage stimulation by insulin reported in a previous paper (2), though statistically significant, was smaller than the degrees of stimulation previously noted by Gemmill and Hamman (6) and by Stadie (7). Even under these improved conditions, it seems probable that insulin added to the medium cannot exert its full effect owing to poor penetration into the central part of the diaphragm.

In view of these considerations, the rates of glucose uptake by diaphragms from normal and from hypophysectomized rats were compared under two sets of conditions. In Series I the conditions were exactly the same as those used for the previous study (2) and permitted a direct comparison of these with previous data. The initial glucose concentration of the medium was 100 mg. per cent, the shaking rate 96 cycles per minute at 2 cm. amplitude, and the time of incubation 2 hours. In Series II the initial glucose concentration was increased to 140 mg. per cent, insulin concentrations of 0,  $10^{-5}$ ,  $10^{-4}$ , and 1 unit per ml. were employed, the shaking rate was 96 cycles per minute at 5 cm. amplitude, and the time of incubation was reduced to 1 hour.

Other manipulations and calculations were carried out as previously described (2). To minimize contamination of control samples with traces of insulin, all glassware and instruments were washed in hot soap or detergent solutions and then dried overnight at 160° before each experiment.

### *Results*

All rates of glucose utilization are expressed as mg. per gm. of wet diaphragm per hour.

The results will be considered under three headings: (a) rate of glucose uptake by diaphragms from normal and hypophysectomized rats in absence of added insulin, (b) concentration of insulin required to produce stimulation

of glucose uptake in diaphragms from normal and hypophysectomized rats, and (c) relative response of diaphragms from normal and hypophysectomized rats to optimum concentrations of insulin.

TABLE I

*Glucose Uptake by Diaphragms from Normal and from Hypophysectomized Rats*

Series I, initial glucose concentration, 100 mg. per cent; 2 hours incubation; insulin concentration, 1 unit per ml. Hypophysectomized animals were fasted 6 hours prior to use, normal animals 18 to 22 hours.

Rat No.	Days after operation	Glucose uptake, mg. per gm. wet tissue per hr.	
		In glucose	In glucose + insulin
1	2	1.7	2.1
2	4	1.9	2.3
3	7	1.7	2.6
4	10	3.0	2.9
5	12	3.3	3.1
6	14	2.3	3.1
7	16	2.5	3.6
8	16	2.1	3.3
9	17	3.1	3.9
10	17	2.3	3.8
11	18	2.3	3.0
12	18	2.9	4.7
13	19	2.5	3.4
14	19	2.4	3.1
15	24	2.3	2.8
16	25	2.3	3.6
17	25	2.3	2.9
18	31	2.3	3.7
Mean for Rats 4-18 (10 or more days after operation)		2.5 $\pm$ 0.08*	3.3 $\pm$ 0.13
Mean for 13 control rats run parallel to hypophysectomized series		1.6 $\pm$ 0.06	2.3 $\pm$ 0.15

Test for significance of stimulation by insulin, hypophysectomized series,  $P = <0.001$ ; control series,  $P = <0.001$ .

Test for significance of difference between hypophysectomized and normal series, no insulin,  $P = <0.001$ ; with insulin,  $P = <0.001$ .

\* Standard error.

*Glucose Uptake without Added Insulin*—In Series I and II the average increases in glucose uptake following hypophysectomy were 56 and 59 per cent respectively. As shown in Tables I and II, these increases were statistically significant.

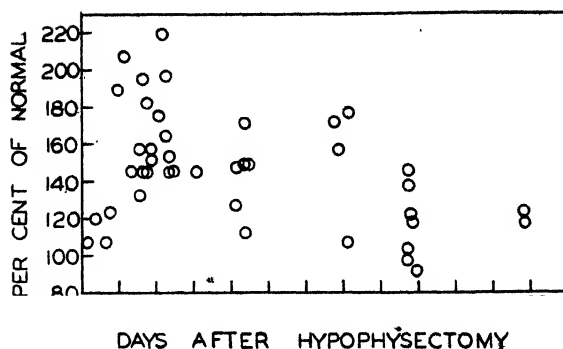


FIG. 1. The glucose uptake without added insulin of diaphragms from rats at various times after hypophysectomy compared on a percentage basis to the average uptake of diaphragms from normal rats.

TABLE II

*Glucose Uptake by Diaphragms from Normal and from Hypophysectomized Rats*

\* Series II, initial glucose concentration, 140 mg. per cent; 1 hour incubation. All animals were fasted 18 to 23 hours prior to use.

Rat No.	Days after operation	Glucose uptake, mg. per gm. wet tissue per hr., at following concentrations of insulin (in units per ml.)			
		0	$10^{-4}$	$10^{-3}$	1
1	8	3.3	2.5	3.4	3.7
2	22	4.7	4.3	4.4	6.2
3	22	5.9	5.0	6.0	6.4
4	23	4.4	4.0		5.6
5	23	5.3	4.6	6.9	
6	24	3.5	2.7	4.4	5.0
7	24	4.1	4.8	4.7	5.6
8	44	4.0	4.4	5.0	6.0
9	44	4.6	4.9	7.1	6.6
10	44	3.0	2.4	4.1	5.7
11	69	4.2	4.1	5.7	4.9
Mean		$4.3 \pm 0.26^*$	$4.0 \pm 0.29$	$5.2 \pm 0.39$	$5.6 \pm 0.27$
Mean for 9 controls run parallel to hypophysectomized series		$2.7 \pm 0.19$	$3.2 \pm 0.28$	$3.9 \pm 0.35$	$4.5 \pm 0.28$

Test for significance of stimulation by insulin. Hypophysectomized series, at  $10^{-4}$ , none; at  $10^{-3}$ ,  $P = 0.07$ ; at 1 unit per ml.,  $P = 0.003$ . Control series, at  $10^{-4}$ , none; at  $10^{-3}$ ,  $P = 0.009$ ; at 1 unit per ml.,  $P = <0.001$ .

Test for significance of difference between hypophysectomized and normal series, no insulin,  $P = <0.001$ ; at 1 unit of insulin per ml.,  $P = 0.01$ .

\* Standard error.

The over-all trend of change in glucose uptake relative to the controls over a period of 4 months after hypophysectomy is shown in Fig. 1. The data suggest that an increased uptake of glucose by diaphragm did not become clearly established until some 10 days after hypophysectomy. At this time a distinct atrophy of the adrenals was demonstrable. In the last group of eleven rats tested 85 to 119 days postoperatively, the average increase in glucose uptake was also slight and not statistically significant.

*Concentration of Insulin Required*—With diaphragms from normal rats,  $10^{-5}$  unit per ml. of insulin produced a marginal, though not significant, stimulation of glucose uptake, and  $10^{-4}$  or 1 unit per ml. produced a significant stimulation (Table II). The marginal concentrations are in agreement with the critical concentrations reported by others (6, 8).

With diaphragms from hypophysectomized animals, on the other hand,  $10^{-5}$  unit per ml. produced no stimulation, and  $10^{-4}$  unit per ml. produced in a few animals a slight stimulation which was not statistically significant for the average of the series. The stimulation by 1 unit per ml. was statistically significant, although there were a few experiments in which the stimulation was small or absent (Table II).

*Effect of Optimum Concentration of Insulin*—The absolute stimulations obtained with high concentrations of insulin were not statistically different in normal and hypophysectomized animals (Tables I and II).

The difference in degree of insulin stimulation between the results of Table I and Table II is ascribed principally to the difference in time of incubation and in degree of agitation of the samples. Statistical analysis shows that insulin stimulation at 1 unit per ml. was significant in both cases.

#### DISCUSSION

The present experiments show that isolated skeletal muscle from hypophysectomized rats uses glucose at a higher rate than normal. This is in agreement with previous observations on intact and on eviscerated hypophysectomized rats (9–11). That insulin still increases the glucose uptake of the diaphragm of hypophysectomized rats is in harmony with the fact that injection of insulin causes hypoglycemia in such animals.

• According to the data of Table II, the diaphragm from a hypophysectomized rat, unlike the whole animal, is not hypersensitive to low concentrations of insulin. This suggests that the hypersensitivity of the fasted hypophysectomized animal is the result of a precarious balance between decreased glucose supply (because of diminished glycogen reserves and limited gluconeogenesis) and increased glucose use. A small amount of insulin, which produces a relatively small increase in glucose uptake in either normal or hypophysectomized rats, may thus precipitate a fatal hypoglycemia in the latter animals.

Since insulin still produces an increase in glucose uptake in diaphragms removed 10 to 119 days after hypophysectomy, it is evident that the hexokinase of muscle is not fully active, even in the absence of pituitary secretions. The source of this non-pituitary inhibition remains to be defined; it may well reside in the structural relationship of hexokinase to other cellular constituents; it may also be dependent on factors conveyed to muscle from tissues other than pituitary.

One of the reasons for working with the isolated diaphragm is to determine the activity of the hexokinase system in a muscle with intact cell structure. The results so far obtained show that the diaphragm from a normal rat has a rate of glucose uptake corresponding to about 50 per cent of the maximum hexokinase activity<sup>4</sup> of that muscle; the corresponding values for diaphragm from diabetic and hypophysectomized rats are about 25 and 75 per cent, respectively. A considerable part of the hexokinase activity of muscle is therefore under hormonal control.

The authors wish to thank Dr. Carl F. Cori for his advice and encouragement during the course of this investigation.

#### SUMMARY

1. The glucose uptake of diaphragms from normal and hypophysectomized rats has been measured. The influence of insulin has also been determined, both on normal diaphragms and on diaphragms taken from rats from 2 to 119 days after hypophysectomy.

2. Diaphragms taken from hypophysectomized rats during the period from 10 to 70 days after operation had rates of glucose uptake which were, on the average, about 60 per cent higher than normal. At less than 10 days or more than 80 days after operation, the difference in rates between the hypophysectomized and the normal groups was less marked.

3. On diaphragms from normal rats,  $10^{-4}$  unit per ml. of insulin produced a significant stimulation of glucose uptake and  $10^{-5}$  unit per ml. produced a marginal stimulation. On diaphragms from hypophysectomized rats neither of these concentrations produced a significant stimulation, while the next higher concentration tested, 1 unit per ml., produced a significant increase of glucose uptake in the majority of the cases. For a few diaphragms from hypophysectomized rats in which the rate without insulin was extremely high, the stimulation by insulin was small or absent.

4. The relation of these findings on isolated muscle to those previously observed on the intact animal is discussed.

<sup>4</sup> In this comparison the maximum rate of hexokinase activity in the diaphragm is the value obtained when insulin is added to a diaphragm taken from a hypophysectomized animal 10 to 70 days after operation.

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# A MICROVOLUMETRIC VAN SLYKE BLOOD GAS APPARATUS\*

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For an analysis of the removal of dissolved  $N_2$  from the blood and tissues of cats (1, 2) a convenient and accurate method for the determination of the  $N_2$  concentration in small amounts of blood was needed. The ingenious syringe micromethod of Edwards, Scholander, and Roughton (3) lacked sufficient sensitivity for part of the study, and it was desirable to have a method requiring less dexterity when large numbers of determinations were to be made. A micro version of the volumetric Van Slyke apparatus was devised in which 0.100 ml. samples of blood could be analyzed with an accuracy of about 0.002 volume per cent. This paper describes this instrument and the procedure for its use.

## *Principle and Design*

In principle the method is that of the macrovolumetric Van Slyke procedure, the vacuum extraction of gases from a known volume of the fluid being analyzed, followed by differential absorption of the gases. The volume of extracted gas is read at atmospheric pressure.

The design of the instrument<sup>1</sup> is shown in Figs. 1 to 4. The lower limit for the bore of the capillary burette (Fig. 1, A, a), used for measuring the volume of the gas bubble extracted from the sample, is set by the facility with which reagents may be drawn through it. 0.25 mm. is a convenient diameter. Uniformity of bore<sup>2</sup> is determined by measuring (to 0.05 mm.) the length of a short thread of mercury at various positions along the length of the capillary; the capillary is calibrated by measuring the length of a long thread of mercury contained in it, and then determining the volume of the mercury from its weight. It is necessary to wet the walls of the capillary with water before filling it with mercury, inasmuch as, in an actual

\* This work has been carried out under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Princeton University.

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<sup>1</sup> The glass blowing was done by Mr. Leigh Harris, Department of Physics, Princeton University, and the machine work by Mr. Russell Mycock, Department of Biology, Princeton University.

<sup>2</sup> The capillary used in the present instrument was selected from stock by the Corning Glass Works.



determination of gas volume, there will be a thin film of water separating the bubble from the glass.

The upper end of the burette, ground flat, protrudes above the water jacket and carries a glass cup (*b*) of 4 or 5 ml. capacity. The cup fits snugly over the end of the capillary and is held in place by stop-cock grease melted and allowed to flow uniformly between the glass surfaces. The total capacity of the instrument to *c*, Fig. 1, *A*, is 0.1000 ml. This pipette is used in measuring the sample of fluid to be analyzed. The bore of the enlargement should not be much greater than 1.5 mm. and the point of its attachment to the capillary burette should be smooth and gradually tapering. A gradual taper is advantageous at the lower end of this enlargement also. The pipette is calibrated, again after wetting its walls, by weighing its capacity of mercury.

The volume from the top of the instrument to the line *d* is about 0.2 ml. Lines *c* and *d* are etched into the glass with hydrofluoric acid; scratching them with a diamond pencil weakens the glass to the extent that it is very likely to break during operation.

The volume of the extraction chamber, *e*, is not critical; in the present instrument it is 8 ml.

The stop-cock, *f*, is a Corning Pyrex, standard taper stop-cock (catalogue No. 7450, 2 mm. bore), reground occasionally with a very fine grinding compound. A precision-ground stop-cock, with a large diameter in order to separate the openings by a greater distance, would be preferable, however. The stop-cock must be carefully ground and greased<sup>3</sup> to insure it against leaking. The arm, *g*, is bent to extend through the aluminum mounting panel. To it is attached a trap and to this, in turn, a good aspirator. The arm leading to the Shohl trap must be bent downward as close to the body of the stop-cock as possible so that no pocket remains in the upper part of the bend that might trap bubbles of gas.

The Shohl trap and mercury leveling bulb are standard. To avoid straining the glass the weight of the mercury-filled rubber tube should be borne by a suitable clamp on the table or other support.

The upper end of the instrument cannot be closed by means of a stop-cock because of the difficulty of accurately calibrating its bore, the possibility of small leaks, and the probability of losing part of the unknown fluid in the irregular crevices. In place of a stop-cock the device shown in Fig. 1, *A*, *i*, and Fig. 1, *B* serves adequately. It consists essentially of a rubber seal seated over the capillary opening by a machine thread arrangement.

<sup>3</sup> The best grease of several tested is the old formula Lubriseal, a brick-red grease of heavy consistency sold by the Arthur H. Thomas Company.

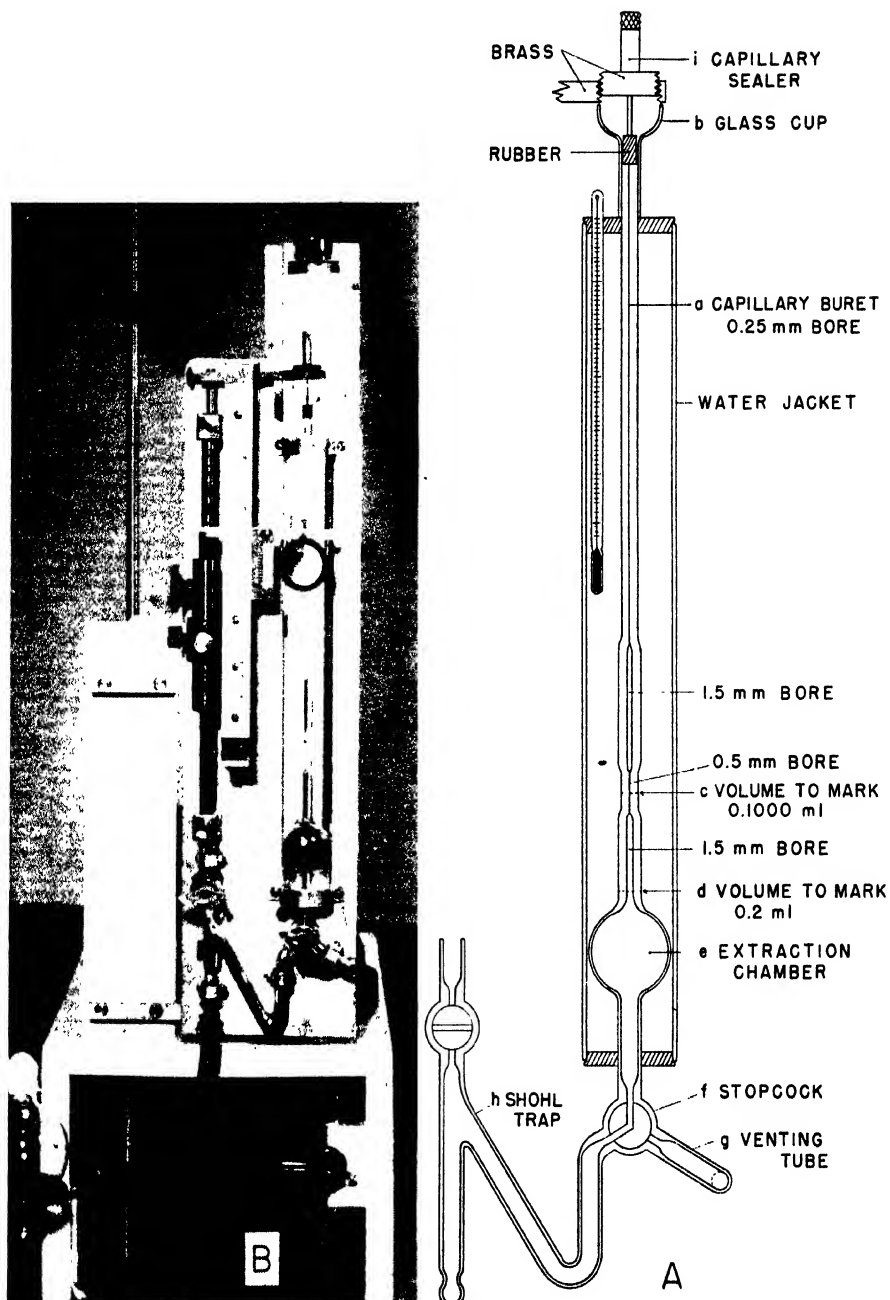


FIG. 1. A, drawing and B, photograph of apparatus

To attain the maximum sensitivity of the instrument a lens to aid in reading the length of the extracted gas column and a device for eliminating parallax are necessary. The device used on the present instrument may be seen in Fig. 1, *B*, and the principle of the antiparallax device is indicated in Fig. 2. A lens, *a*, is mounted in front of the capillary burette so that a meniscus, *b*, within the capillary is in focus. A cross-hair, *c*, made of a fiber of very fine glass wool, is mounted close behind the lens and at right angles to the length of the capillary. Exactly half-way between the cross-hair and the lumen of the capillary a small mirror, *d*, is mounted, facing the lens. While one observes the meniscus through the lens from directly in front of the instrument, the mirror is brought into view from one side until its leading edge just begins to encroach on the image of the meniscus.

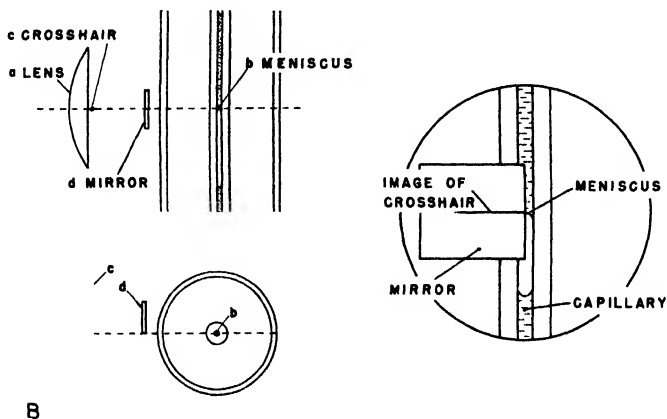


FIG. 2. Cross-hair and antiparallax device. *A*, lateral section; *B*, cross-section; *C*, view of meniscus and image of cross-hair through lens.

With the mirror correctly placed, one can see in it the image of the cross-hair projected onto the magnified view of the meniscus, with no parallax, as in Fig. 2, *C*.

This whole assembly is mounted on a brass arm in such a fashion that each element is adjustable. Also carried on this arm is a metric scale vernier, reading to 0.05 mm. The arm is rigidly attached to the movable part of a rack and pinion from a discarded microscope for fine adjustments. This, in turn, is carried by means of a brass block on a  $\frac{3}{8}$  inch drill rod, mounted vertically on the aluminum panel, for coarse adjustment. The block is held in position and prevented from swinging in an arc on the drill rod by a set screw that fits into a channel milled along the length of the rod.

A steel metric rule is mounted on the aluminum panel parallel to the cap-

illary burette and in such a position that the vernier is juxtaposed to the scale along its entire length. The zero mark of the scale is at the bottom. By means of this assembly it is a simple matter to repeat measurements to 0.05 mm. anywhere along a linear distance of 20 cm.

The instrument and measuring device are carefully mounted on a 3/16 inch aluminum panel, cushioned at the points of contact by rubber. A rectangular area is cut out of the panel behind the capillary burette and extraction chamber to permit illumination of the apparatus by transmitted light. The yoke that carries the seal for the upper end of the capillary is held in place on the panel by means of a set screw so that it may be conveniently removed.

To lessen strain, the apparatus is shaken during an extraction by swinging the panel through a short arc in the fashion of a door, rather than by rocking the top forward and backward as is customary with the macro Van Slyke apparatus. The panel is pivoted through a distance of 1 or 1½ inches at a rate of about 250 cycles a minute.

The extraction chamber and pipette are illuminated from behind by either a fluorescent bulb or an incandescent one. In the latter case the bulb must be some distance in back of the apparatus in order not to heat it, and even then a heat-absorbing glass filter such as Sollex<sup>4</sup> is desirable.

In the determination of dissolved N<sub>2</sub> in blood, the analysis for which the instrument was originally designed, a number of solutions and accessory devices are required. Since, for the most part, these devices or modifications of them would also be needed for any other determination for which the instrument might be used, they are described here.

A gas-free solution of sodium hydrosulfite and sodium anthraquinone- $\beta$ -sulfonate in KOH (15 gm. of a 10:1 mixture of the salts in 50 ml. of 1 N KOH) is required for the absorption of O<sub>2</sub> and CO<sub>2</sub>, as in the standard macromethod (4). The solution is degassed by shaking approximately 20 ml. of it in the extraction chamber of a standard manometric Van Slyke apparatus or similar device. The simplest and most effective method is to connect a good water aspirator with the extraction chamber through the exit tube at the top, after evacuating the extraction chamber in the usual fashion with the mercury leveling bulb. About 10 minutes of vigorous shaking will degas the solution more effectively than will a large number of repeated extractions.

Once degassed, this solution is stored anaerobically in a 10 ml. hypodermic syringe lubricated thoroughly with glycerol. The syringe is filled from the side tube at the top of the extraction chamber through a short length of rubber tubing.

To transfer the solution anaerobically to the extraction chamber of the

<sup>4</sup> Obtained from the Pittsburgh Glass Company, Philadelphia.

micro Van Slyke apparatus an adapter similar to that shown in Fig. 3 is attached to the syringe (neither the piece of steel tube cemented in the needle socket nor the bend in the needle shaft is needed for this adapter). Solution is transferred from the syringe to the apparatus by holding the gasket of the adapter firmly to the upper end of the capillary burette, opening the lower stop-cock (with the mercury leveling bulb in the lower position), and expelling the fluid from the syringe. Solutions stored in a syringe in this fashion will be maintained in a gas-free condition for a very long time if the tip of the needle is kept immersed in mercury while not being used.

The only other reagent required for  $N_2$  determinations is caprylic alcohol, to prevent foaming of the blood. This is carried in a 1 ml. syringe fitted

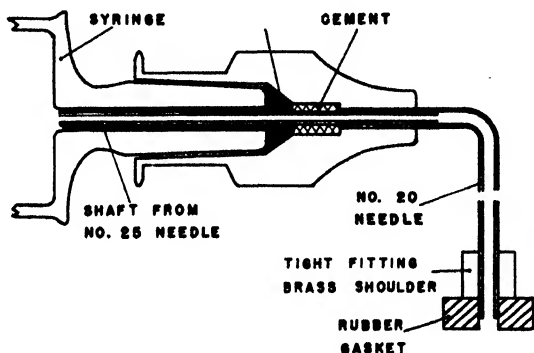


FIG. 3. Hypodermic needle adapter for transferring blood anaerobically from the collecting syringe to the apparatus.

with a hypodermic needle adapter. The alcohol is not degassed prior to an analysis.

A third 5 ml. syringe for mercury is fitted with either a regular hypodermic needle or a glass pipette tip attached with rubber tubing.

#### *Procedure for $N_2$*

For non-protein fluids which do not form a gummy coagulum with the reagents, no more than 10 minutes are required for a determination. About 15 minutes are needed for blood because of the greater difficulty in cleaning the apparatus.

1. The clean apparatus is filled with mercury from the leveling bulb, and a good vacuum is established in the side arm of the 3-way stop-cock (Fig. 1, A, g).

2. About 0.15 ml. of distilled water is drawn into the chamber from the cup, followed by mercury to displace the water from the pipette at least as far as the 0.1 ml. mark.

3. The seal is screwed firmly in place to close the capillary burette, and the extraction chamber is evacuated by means of the leveling bulb. A small amount of mercury is left in the chamber to aid in agitating the water during the subsequent extraction.

4. The water is extracted with vigorous shaking for a minute.

5. The shaker is stopped, the leveling bulb lowered further than was required to evacuate the chamber in step (3), and the 3-way cock turned to connect the chamber with the leveling bulb. The gas-free water will be carried down into the stop-cock and the upper arm of the Shohl trap, dislodging any tiny bubbles of gas that may have been trapped there. This step is essential.

6. Mercury is readmitted into the chamber and the extracted gas and water are ejected.

7. A little more than 0.2 ml. of the alkaline hydrosulfite solution is run into the pipette from the storage syringe. There should be some water in the cup during this operation.

8. About a cm. of caprylic alcohol is run into the capillary burette.

9. Mercury is added to the upper cup and slowly drawn into the apparatus to clear the capillary and pipette of reagents at least as far as the 0.1 ml. mark. It is essential for this step that the design and dimensions of the pipette be as specified earlier; otherwise the mercury will round up and drop freely through the enlarged part without clearing the hydrosulfite from the pipette. If the pipette is correctly made, the mercury will expand completely into the enlargement, forcing the hydrosulfite ahead of it.

10. The capillary is sealed and the chamber evacuated by the leveling bulb. If gas has not appeared in the chamber by the time the leveling bulb has reached the appropriate level, a light tap delivered to the top of the capillary seal will cause the mercury column to "break" directly under the seal, clearing the pipette of mercury and hydrosulfite.

11. Extraction is effected by shaking for 2 minutes. 1 minute is adequate, but 2 minutes are used for safety.

12. Mercury is readmitted to the chamber smoothly until the small bubble is in the capillary. The leveling bulb is held a cm. or two above the level of mercury in the chamber, the capillary is opened, and the bubble expelled from the apparatus along with hydrosulfite until the volume remaining in the apparatus is just 0.2 ml.

13. Step (9) is repeated.

14. The sample to be analyzed is admitted anaerobically to the pipette from the collecting syringe to the 0.1000 ml. mark by means of a needle adapter fitted with a rubber gasket, as with the hydrosulfite.

15. Steps (9), (10), and (11) are repeated.  $O_2$  and  $CO_2$  extracted from

the blood are absorbed by the alkaline hydrosulfite, and  $N_2$ ,  $CO$ ,<sup>5</sup> and the inert gases are extracted nearly completely into the free gas space.

16. Mercury is carefully readmitted to the extraction chamber from the leveling bulb until the liquid has risen into the capillary burette about 1 or 2 cm. The stop-cock is closed and the stopper sealing the capillary is opened, letting clean water from the cup down into the capillary on top of the extracted gas. By careful manipulation of the stop-cock and the leveling bulb, a small amount of water is drawn through the burette to wash it. During this, the bubble of  $N_2$  is drawn into the pipette enlargement. Care should be taken to avoid breaking the bubble into smaller ones because it is sometimes difficult to make these coalesce, especially in protein solutions. If this does occur, the bubbles may be caused to coalesce by forcing them up into the capillary, or in any event by drawing a droplet of caprylic alcohol down on top of them. Washing can only be done with relatively insoluble gases such as  $N_2$  and even then excessive washing should be avoided.

17. The bubble is run up into the capillary burette to a convenient level for reading. The upper limit of the gas column should always be brought to approximately the same level.

18. The length of the column is measured by reading, in quick succession, the scale settings with the cross-hair at the top and bottom of the column. For maximum accuracy several measurements are averaged, being read alternately from the top to bottom and from bottom to top of the column. Readings on a bubble may easily be repeated to 0.05 mm.

19. The barometric pressure and the temperature of the water jacket at the time of measurement are read.

20. The instrument is cleaned for the next analysis. Solutions such as water, which form no precipitate with the hydrosulfite, are simply ejected through the capillary and drawn off from the cup by a suction tube. The apparatus is then ready for the next analysis. Steps (2) through (6) are omitted. With proteinaceous fluids, however, a gummy precipitate<sup>6</sup> forms which cannot be passed through the capillary. The mercury in the extraction chamber is run into the leveling bulb, and as the last of it reaches the stop-cock the cock is turned to connect the chamber with the clean-out side arm (Fig. 1, A, *g*). The contents of the chamber are sucked through it into the trap in the vacuum line, and the apparatus is cleaned by sucking first a detergent<sup>7</sup> and finally distilled water through it. After rinsing, the stop-

<sup>5</sup> If  $CO$  is present in significant amounts, it is measured and corrected for by absorbing it with Winkler's reagent (4).

<sup>6</sup> The formation of this precipitate is markedly reduced if potassium hydroxide is used in preparing the hydrosulfite solution rather than sodium hydroxide.

<sup>7</sup> The neutral detergent Drene, diluted 50 per cent with water, is very satisfactory.

cock is closed and a vacuum reestablished in the clean-out side arm. By maintaining a vacuum in this arm the danger of air leaking from it into the extraction chamber during an analysis is eliminated.

*Calculations*—Concentration of  $N_2$  is calculated from the following equation.

$$\text{Volumes } \% = \frac{\text{length of gas column in mm.} \times a \times b \times 100}{100 \text{ microliters}}$$

100 microliters is the volume of the fluid analyzed.  $a$ , the number of microliters per ml. of capillary, is the calibration factor of the capillary burette. The factor  $b$  corrects the volume of moist gas at room temperature and pressure to the volume of dry gas at standard conditions (determined from Table 15, p. 129, of the text of Peters and Van Slyke (4)). However, there are two corrections which must be made to the apparent barometric pressure in order to determine the actual gas pressure inside the gas column. One of these results from the hydrostatic pressure of the column of water in the capillary above the gas bubble, equal to the length of the column of water divided by the density of mercury, 13.5. This pressure is added to the barometric pressure. The second correction results from the inwardly directed pressure due to surface tension at the two menisci of the gas column. In a capillary of small diameter this may become very appreciable. Its magnitude may be calculated from the relation,  $p = 2\gamma/r \times 7.501 \times 10^{-4}$ , where  $p$  is the pressure, in mm. of mercury, within a gas bubble of radius of curvature  $r$  cm., in a liquid whose surface tension at the gas-liquid surface is  $\gamma$ . The constant converts dynes per sq. cm. to mm. of mercury. This relation is applicable to the present calculation because, to a first approximation, the radius of curvature of a meniscus in a capillary of small diameter, and when the contact angle is zero, is the same as the radius of the capillary. The two menisci are, then, hemispheres, and together constitute a sphere. The liquid is assumed to be pure water, though undoubtedly some surface tension-lowering impurities are present. In the present instrument this correction amounts to 7.4 mm. of mercury, which must also be added to the barometric pressure.

The  $c$  correction of Peters and Van Slyke, used to make allowance for the gas unextracted from the liquid phase when equilibrium has been reached, is negligible (0.06 per cent) in the present instrument for the determination of  $N_2$ , because of the much larger ratio of volume of the fluid phase to volume of the extraction chamber in the micro instrument than in the macro-manometric apparatus.

Reabsorption of gas during the process of reestablishing atmospheric pressure after extraction and during the time required to read the gas volume is negligible in this instrument, as in the macrovolumetric instrument,



if the gas is  $N_2$ . Repeated washing of the extracted  $N_2$  bubble with distilled water does not appreciably alter its volume.

Another small correction results from the hemispherical nature of the meniscus at the ends of the gas cylinder. In measuring the length of the bubble the cross-hair is set at the bottom of the meniscus, the fact that the meniscus is curved being neglected. The error is constant for all measurements and, if of sufficient magnitude, the volume of gas as measured can be corrected for it before reducing it to the volume at standard conditions. In the present instrument it is of approximately the same magnitude as the over-all error of the method.

*Sensitivity and Accuracy of Method*—The limit of sensitivity of the present instrument is 0.0027 microliter or  $1.20 \times 10^{-10}$  mole of gas. This corres-

TABLE I  
*Determination of Dissolved  $N_2$  in Distilled Water*

Five analyses were made of each sample.

$N_2$ concentration		Standard error
Calculated	Determined	
<i>vol. per cent</i>	<i>vol. per cent</i>	<i>vol. per cent</i>
1.158	1.159	$\pm 0.0005$
0.576	0.576	$\pm 0.0008$
0.571	0.575	$\pm 0.0007$
0.457	0.458	$\pm 0.0007$
0.356	0.365	$\pm 0.0004$
0.135	0.137	$\pm 0.0010$

ponds to a difference in gas bubble length of 0.05 mm. in the capillary burette.

To determine the accuracy of the instrument and its reproducibility on repeated analyses of the same solution, analyses of water containing known amounts of  $N_2$  were made. Two lots of water were equilibrated in tonometers, one with air and the other with oxygen of known  $N_2$  content, and the  $N_2$  concentrations in the samples calculated from the absorption coefficient and tension of  $N_2$  in the tonometer. Water with intermediate concentrations was prepared by mixing known amounts of these two samples in a 10 ml. syringe.

It is evident from the results of these test analyses, summarized in Table I, that both the absolute accuracy and the reproducibility are quite high regardless of whether the concentration of  $N_2$  in the sample is high or very low, and are equal to the limit of sensitivity of the measuring device.

For these determinations the apparatus was not cleaned between analyses by the method described in step (20). To determine whether the cleaning

procedure adversely affects the reproducibility of the method, a sample of blood was analyzed ten times with the cleaning technique. The mean concentration of the ten determinations, with the standard error, was  $1.009 \pm 0.0006$  volumes per cent, giving the same reproducibility as with the water samples.

*Blood Sampling*—In the study of denitrogenation of cats (1, 2) two methods of obtaining blood samples were used. For larger vessels such as the postcaval vein and the dorsal aorta 1 ml. syringes and No. 26 needles were used. For very small blood vessels, such as drain a single muscle or a fat deposit in the cat, the syringe was unsatisfactory because of the difficulty in piercing the small vessels with a hypodermic needle and the long time required to obtain suitably large samples. In its place a method with use of a glass sampling tube has been devised.

The following technique is employed with syringes. The syringe is first thoroughly wet with water. A little Carbowax, No. 1500,<sup>\*</sup> is placed on the ground tip of the syringe, the needle is firmly seated in place, and the wax is melted to effect an air-tight seal. Bubbles are then removed from the syringe and needle, and the syringe is rinsed two or three times with an anticoagulant solution (5 per cent sodium oxalate and 1.5 per cent sodium fluoride), air bubbles being avoided. Glycerol is applied liberally to the socket at the top of the syringe so that it will be carried down between the barrel and plunger. A gas-free solution of the anticoagulant is prepared in a standard manometric Van Slyke apparatus as described above for sodium hydrosulfite. A short piece of gum rubber tubing with a pinch-cock at one end is attached to the side arm of the extraction chamber. Some of the gas-free solution is washed through this and the pinch-cock is closed. The anticoagulant (not degassed) in the syringe is ejected completely, the rubber tubing punctured, and the gas-free solution drawn into the syringe to rinse the needle and syringe thoroughly. About 0.2 ml. of the anticoagulant is left in the syringe to be ejected immediately before introduction into the blood vessel. The amount of oxalate left in the socket of the needle and on the walls of the syringe will prevent clotting in 1 ml. of blood.

The use of gas-free anticoagulant prevents contamination of the sample with an unknown amount of  $N_2$ , but a correction must be made for the dilution of the blood by the anticoagulant solution. For this the volume of the dead space of the syringe and needle must be known, and the syringe should be recalibrated at the 1 ml. mark. These volumes are calculated from the weights of the syringe and needle when dry, filled completely with water to the 1 ml. mark, and after the water has been ejected. Among twenty-three syringes, the greatest variation in volume at 1 ml. was 0.028 ml. The

<sup>\*</sup> Obtained from the Carbide and Carbon Chemicals Corporation, 30 East 42nd Street, New York.

volume of the dead space varied between 0.044 and 0.036 ml., with a mean of 0.040 ml. The variation in the dead space of needles of the same size is not great.

To seal the syringe after drawing the sample, the needle is removed and about 0.05 ml. of mercury is drawn into the syringe, the tip of which is then closed with the finger. The blood is thoroughly mixed by rolling the drop of mercury back and forth in the syringe, and is stored by placing the syringe tip down in a beaker of ice water containing about a half inch of mercury. Glycerol is again applied to the top of the syringe between the barrel and the plunger to improve the seal and prevent freezing of the syringe.

The blood is transferred to the analyzing apparatus through the special needle adapter shown in Fig. 3. When attaching this adapter to the syringe, the socket is first filled with mercury and the syringe is then introduced, displacing the mercury as it is seated. The sample is thoroughly mixed and some of it is ejected through the adapter to clear it of air. The design of the adapter and the precautions in its use eliminate air that might otherwise be trapped in the socket of the adapter and contaminate the sample. Without the smaller tube that projects into the syringe tip, erratic results in repeated analyses of a sample are frequently obtained. The shaft is bent at right angles to prevent mercury from passing into the analyzing apparatus during transfer.

The glass capillary sampling tube used for small blood vessels is shown in Fig. 4, *A*. The fine, sharply beveled tip is comparatively easy to insert into even a small vein. It contains 0.15 to 0.2 ml. of blood. The tip offers the only problem in the construction. It is made, after the two tapers have been drawn in the glass, by holding the tip lightly against the side of a small motor-driven grindstone of the type used in dental grinding apparatus. A very good bevel can be made on microscopically fine tips in this way if the motor is fairly vibration-free and the stone of fine grade.

In preparing the capillary for use the device shown in Fig. 4, *B* is convenient. The tip of a clean, dry capillary is inserted through a pinhole in the rubber sheet and down into the mercury. A few mm. of mercury are drawn into the capillary. The tip is lifted into the saturated,  $N_2$ -free<sup>9</sup> sodium oxalate and an equal amount of this is drawn up, followed finally by another mercury seal. The sharp tip of the capillary is then forced through the vein wall and a sample slightly in excess of 0.1 ml. is drawn up, carrying the first two mercury seals and the anticoagulant ahead of it. Another mercury seal is taken into the tip after the blood. With a finger

<sup>9</sup>  $N_2$  is removed from the solution by bubbling pure  $O_2$  through it for 1 or 2 hours. The solution is transferred by means of a syringe, and maintained  $N_2$ -free by keeping it under  $O_2$ .

held over the open back end of the capillary, a tiny bead of mercury is shaken loose from this last seal and is caused to roll back and forth through the blood to mix it thoroughly with the anticoagulant which has wet the walls. The beveled tip is then sealed in a micro flame and the tube stored in a refrigerator until analyzed. Samples are analyzed as soon as possible because the amount of anticoagulant is barely sufficient to prevent coagulation.

In transferring the sample from the capillary to the analyzing apparatus, the blood is first thoroughly mixed with the aid of the droplet of mercury. The tip of the capillary is then cut off with a carborundum point about 2 mm. from the shoulder at the start of the first taper, and a rubber gasket of the type used on the needle adapters is pushed onto the tip up to this

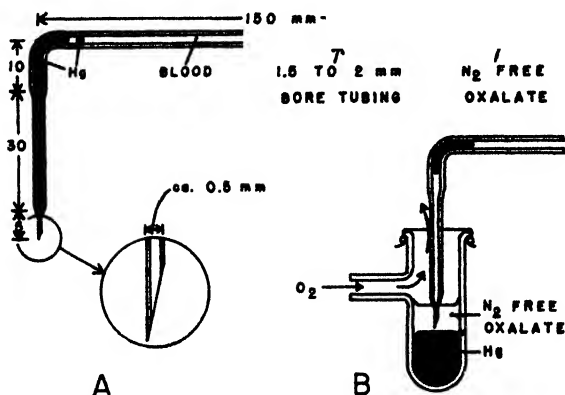


FIG. 4. A, glass capillary tube for sampling small blood vessels; B, device for charging the tube with  $N_2$ -free anticoagulant.

shoulder. The mercury seal is then forced out by carefully applied pressure and the sample is transferred in the usual way.

*Analysis of Other Gases*—Although little experience has been had in using this instrument with gases other than  $N_2$ , there should be no difficulty in adapting it to the relatively insoluble gases such as  $O_2$  and  $CO$ . For measuring the  $O_2$  content of blood it would be advantageous to use a considerably smaller sample of blood than 0.1 ml. in order that the quantity of  $O_2$  released will not exceed the capacity of the capillary burette (0.01 ml. would yield a column of  $O_2$  5 cm. long in the burette). A sample of any size can be used in the instrument if the pipette is calibrated at regular intervals, measured by the cross-hair device.

In the analysis of very soluble gases such as  $CO_2$  the instrument suffers by comparison with the standard manometric Van Slyke apparatus in that the final measurement is made at 1 atmosphere of pressure, at which the tendency for resolution of the gas is greater. Even then, preliminary

experience with saturated salt solutions to lessen the solubility of  $\text{CO}_2$ , and saturated sodium monobasic phosphate in place of lactic acid to liberate  $\text{CO}_2$  from carbonate, gives hope for reasonable accuracy on samples of plasma of only 3 or 4 microliters (measured in the capillary burette).

It is probable that any analysis that can be performed on the macro Van Slyke apparatus can also be carried out with the micro instrument, frequently with an increase in accuracy and a decrease in time.

It is a pleasure to acknowledge the interest and many helpful suggestions of Professor E. Newton Harvey and Dr. William D. McElroy during the development of this apparatus.

#### SUMMARY

A microvolumetric Van Slyke apparatus for the determination of minute amounts of dissolved gases has been devised by which differences in gas volume of 0.0027 microliter, or  $1.20 \times 10^{-10}$  mole, of gas can be measured. The procedure is described for the determination of dissolved  $\text{N}_2$  in 0.100 ml. of fluid. 10 to 15 minutes are required, and analyses may be repeated with a standard error of about  $\pm 0.0008$  volume per cent on samples varying in concentration from 1.0 to 0.1 volume per cent. Applicability to other determinations is discussed.

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# THE ENZYMATIC MECHANISM OF OXIDATION-REDUCTIONS BETWEEN MALATE OR ISOCITRATE AND PYRUVATE\*

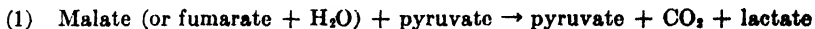
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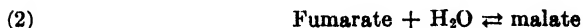
The discovery of carbon dioxide assimilation by heterotrophic bacteria (1) and animal tissues (2) aroused considerable interest in the mechanisms involved. Evidence from various sources suggested the Wood and Werkman reaction, *i.e.* the carboxylation of pyruvate to oxalacetate, as a major pathway (3).

Evans, Vennesland, and Slotin (4) discovered that pigeon liver extracts fixed  $\text{CO}_2$  in the presence of malate (or fumarate), pyruvate, and catalytic amounts of manganous ions and of either diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN), while catalyzing the overall Reaction 1.



This is essentially a dismutation between malate, which is oxidized and decarboxylated to pyruvate +  $\text{CO}_2$ , and pyruvate, which is reduced to lactate; it will be referred to as the malate-pyruvate dismutation.

Further work (4, 5) suggested that Reaction 1 is the net result of the following reactions.



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Reversibility of the above reactions was indicated by the incorporation of isotopic  $\text{CO}_2$  in the carboxyl groups of the various acids (6). Reactions 2, 3, and 5 are catalyzed by the enzymes fumarase, malic dehydrogenase, and lactic dehydrogenase respectively, which are present in large amounts in pigeon liver extracts. An enzyme catalyzing Reaction 4 from left to right, in the presence of  $\text{Mn}^{++}$ , was discovered in *Micrococcus lysodeikticus* by Krampitz and Werkman (7) and in pigeon liver extracts by Evans *et al.* (4).

Since it should be possible to study the combined result of Reactions 3 and 4, *i.e.* Reaction 6, by spectrophotometric methods as in the case of the isocitric dehydrogenase-oxalosuccinic carboxylase system (8), attempts

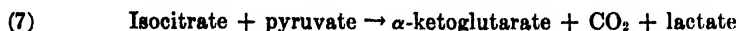


were made to reconstruct Reactions 1 and 6 with a combination of purified enzymes. The enzymes used were malic dehydrogenase (9), crystalline lactic dehydrogenase (10), and a partially purified oxalacetic carboxylase from *Micrococcus lysodeikticus*, the preparation of which will be described in this paper. The results were completely negative.

Further investigation of the properties of pigeon liver extracts and of ammonium sulfate fractions thereof showed that Reaction 1 specifically requires TPN as coenzyme. These observations led to the discovery of an enzyme in pigeon liver which, with TPN as coenzyme, catalyzes the reversible oxidative decarboxylation of *l*-malic acid to pyruvic acid and  $\text{CO}_2$  (11).

The purification and properties of the new enzyme are described in the following paper (12). The purified pigeon liver enzyme, which still contains some lactic dehydrogenase, catalyzes Reaction 1, in the presence of  $\text{Mn}^{++}$  and TPN, with *l*-malate and pyruvate as initial reactants; addition of crystalline lactic dehydrogenase accelerates the rate of this reaction.

Moulder, Vennesland, and Evans (5) described a reaction between isocitrate and pyruvate, yielding  $\alpha$ -ketoglutarate,  $\text{CO}_2$ , and lactate (Reaction 7) catalyzed by pigeon liver extracts in the presence of  $\text{Mn}^{++}$  and either



DPN or TPN. It is essentially a dismutation whereby isocitric acid is oxidized and decarboxylated to  $\alpha$ -ketoglutarate and  $\text{CO}_2$  while pyruvate is reduced to lactate, and will be referred to as the isocitrate-pyruvate dismutation. This reaction, which should be catalyzed by the enzymes isocitric dehydrogenase, oxalosuccinic carboxylase, and lactic dehydrogenase, was reported to occur only in pigeon liver extracts or after supplementing other

tissue preparations with fractions from pigeon liver (5). Since the muscle and heart preparations used contain the above enzymes (5, 13), it was desirable to investigate this problem further.

We found that the isocitrate-pyruvate dismutation readily occurs in extracts of washed, acetone-dried, pig heart (which contains the isocitric and oxalosuccinic enzymes but little or no lactic dehydrogenase) when supplemented with crystalline lactic dehydrogenase,  $Mn^{++}$ , and TPN.

Here again, as would be expected from the TPN-specificity of isocitric dehydrogenase (8, 14), the reaction is strictly TPN-specific and this is true also if pigeon liver preparations are used.

In the course of this work it has been found that crude pigeon liver preparations catalyze the conversion of DPN to TPN in the presence of adenosine triphosphate (ATP). Such a conversion had been previously observed by Adler *et al.* (15) in yeast Lebedev juice. This finding may help to explain the difficulties in demonstrating the true pyridine nucleotide specificity of the above dismutations.

Since the reductant system in both the malate-pyruvate and the isocitrate-pyruvate dismutation is TPN-specific, whereas the oxidant system, lactic dehydrogenase, was considered to be DPN-specific, there remained the question of how the electron transfer between the two systems takes place. This was investigated by Moulder *et al.* (5) who reached the conclusion that the lactic (and also the malic) dehydrogenase of pigeon tissues can react with either DPN or TPN.

On reinvestigating the pyridine nucleotide specificity of various dehydrogenases, by direct spectrophotometric measurement of the oxidation or reduction of the nucleotide, we found that lactic and malic dehydrogenases from various sources, whether crude or pure, can indeed react with either DPN or TPN, although the reaction with the former is much faster. Glutamic dehydrogenase (liver) was found, in confirmation of von Euler *et al.* (16), to react equally fast with either nucleotide. Crystalline triose phosphate dehydrogenase (17) was found to be strictly DPN-specific, whereas isocitric dehydrogenase (pig heart, pig liver, pigeon liver) is strictly TPN-specific.

#### EXPERIMENTAL

*Malate-Pyruvate Dismutation*—Catalysis of this dismutation by dialyzed aqueous extracts of acetone powder of pigeon liver is shown in Fig. 1 (Curves 1, 2, 4). Preparation of the extracts and experimental conditions were similar to those described by Moulder *et al.* (5). Fig. 1 also shows that the pigeon liver enzymes cannot be replaced by mixtures of purified malic dehydrogenase, oxalacetic carboxylase, and lactic dehydrogenase (Curve 3), containing these enzymes in much higher quantities than those



present in effective amounts of pigeon liver extract. Not only is there no reaction with the mixture of purified enzymes, but the addition of such

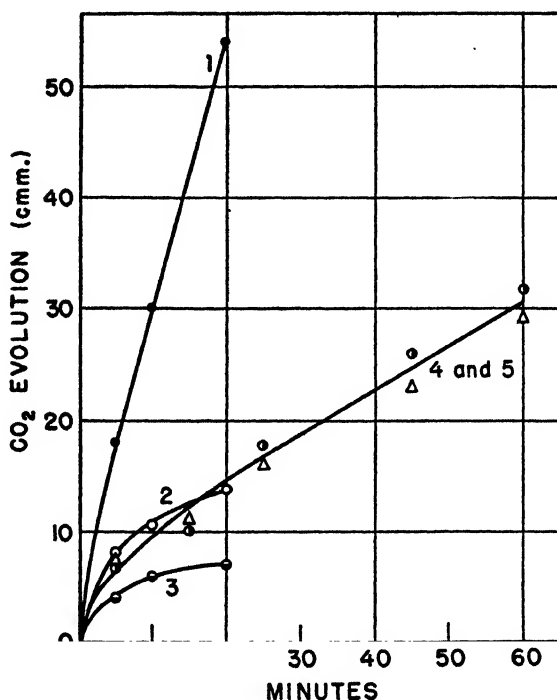


FIG. 1. Failure of purified malic dehydrogenase, oxalacetic carboxylase (from *Micrococcus lysodeikticus*), and lactic dehydrogenase to catalyze the malate-pyruvate dismutation. 0.05 M acetate buffer, pH 5.2, 5 micromoles of  $MnCl_2$ , 0.106 micromole of DPN, 10 micromoles of *l*-malate (224 c.mm.), and 48 micromoles of pyruvate with other additions as indicated. Final volume 2.4 cc. Gas, air. Temperature, Curves 1, 2, and 3, 38°; Curves 4 and 5, 30°. The reaction was started by tipping in substrates from the side bulbs of Warburg vessels after temperature equilibration. Curve 1, 1 cc. of dialyzed pigeon liver extract with about 30 mg. of protein. Curve 2, 0.2 cc. of pigeon liver extract. Curve 3, pure malic dehydrogenase (14,000 units), purified oxalacetic carboxylase from *Micrococcus lysodeikticus* (200 units), and crystalline lactic dehydrogenase (40,000 units). Curve 4 (●), 0.5 cc. of pigeon liver extract. Curve 5 (Δ), 0.5 cc. of pigeon liver extract, malic dehydrogenase (5600 units), oxalacetic carboxylase (200 units), and crystalline lactic dehydrogenase (20,000 units). 1.0 cc. of pigeon liver extract contained 3600 units of malic dehydrogenase, 160 units of oxalacetic carboxylase, and 2400 units of lactic dehydrogenase.

mixtures to pigeon liver extract does not affect the rate of dismutation obtained with the latter alone (Fig. 1, cf. Curves 4 and 5). In order to check on the possibility of inactivation of the purified enzymes during the experiment, suitable aliquots of the reaction mixtures were assayed for the various

enzymes at the end of the manometric runs; recoveries between 50 and 100 per cent were obtained.<sup>1</sup>

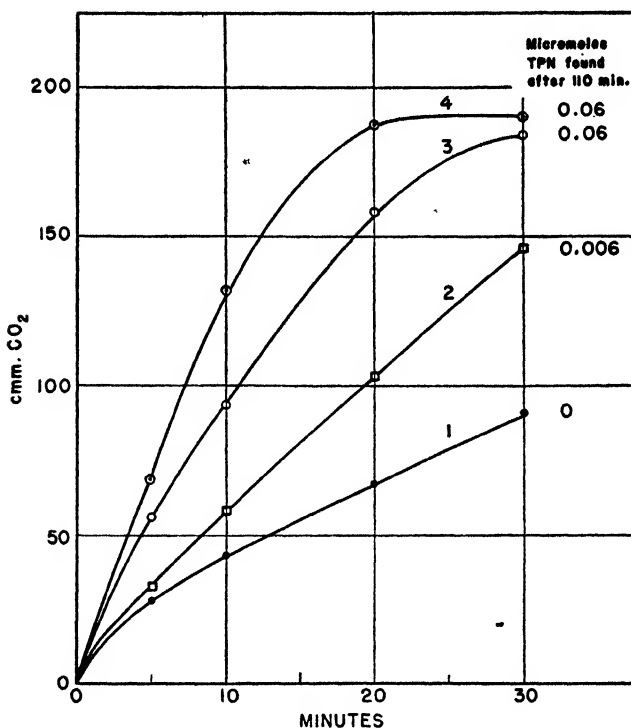


FIG. 2. Malate-pyruvate dismutation with crude pigeon liver enzymes. The enzyme was prepared by precipitating an aqueous extract of acetone-dried pigeon liver with saturated ammonium sulfate at 0°. The precipitate between 0.38 and 0.65 saturation was dissolved in a minimum volume of water and the solution was dialyzed at 3–4° for 15 hours against 0.025 M phosphate buffer, pH 7.4, 0.1 M acetate buffer, pH 5.2, 5 micromoles of  $MnCl_2$ , 8.5 micromoles of *l*-malate (190 c.mm.), 50 micromoles of pyruvate, and 0.5 cc. of enzyme with other additions as indicated. Final volume 2.0 cc. Gas, air; temperature, 38°. The reaction was started by tipping in substrates from the side bulbs of Warburg vessels after temperature equilibration. Curve 1, either no further additions or 5 micromoles of ATP; Curve 2, 0.5 micromole of DPN; Curve 3, 0.5 micromole of DPN and 5 micromoles of ATP; Curve 4, 0.135 micromole of TPN.

On reinvestigation of the pyridine nucleotide specificity of the malate-pyruvate dismutation it became apparent that, even after prolonged dialysis, extracts are unsuitable for this purpose because there is little

<sup>1</sup> For a description of the enzyme tests and definition of units see the section "Methods."

stimulation of the reaction rate by pyridine nucleotides beyond that caused by  $Mn^{++}$  (*cf.* also Moulder *et al.* (5)). A dialyzed ammonium sulfate fraction, prepared from the extracts by precipitation between 0.38 and 0.65 saturation, proved much more satisfactory. When this preparation is used in place of the extracts, although the basal rate of dismutation on addition of  $Mn^{++}$  alone is not insignificant, there is not much further stimulation by DPN. Addition of TPN accelerates the reaction very markedly (Fig. 2), suggesting that the malate-pyruvate dismutation is TPN-specific.

If a combination of ATP and DPN is substituted for TPN, one obtains dismutation rates not much below those obtained with TPN itself. It

TABLE I

*Enzymatic Synthesis of TPN from DPN and ATP in Ammonium Sulfate Fractions of Pigeon Liver*

Enzyme preparation, composition of reaction mixtures, and experimental conditions as in Fig. 2. Experiments 1 and 2 were carried out with preparations from different livers.

Experiment No.	Nucleotide added			Incubation time	TPN found
	ATP	DPN	TPN		
	micromoles	micromole	micromole	min.	micromole
1				110	0
	5			110	0
		0.5		110	0.004
	5	0.5		110	0.04
			0.135	110	0.04
			0.135	0	0.087
2				70	0.005
	5			70	0.008
		0.5		70	0.008
	1.25	0.5		50	0.024
	2.50	0.5		50	0.037
	5	0.5		70	0.087
			0.135	70	0.061

could be shown that, under these conditions, there occurs a synthesis of TPN; this is illustrated in Table I in which Experiment 1 corresponds to that of Fig. 2. The TPN estimations were carried out as described under "Methods." It may be seen (Table I, last sample of Experiment 1) that on boiling a sample immediately after addition of 0.135 micromole of TPN only 0.087 micromole, or about 65 per cent, was recovered, the remainder being probably carried down by the heavy protein precipitate. Hence a correction for recovery was applied to the values for found TPN given in Fig. 2. It will also be observed (Table I) that added TPN disappears on incubation; whether by degradation to DPN or otherwise is unknown.

As already stated, the above results led to the isolation from pigeon liver of a TPN-specific enzyme which catalyzes the oxidative decarboxylation of *l*-malic acid to pyruvic acid and  $\text{CO}_2$ . The malate-pyruvate dismutation is catalyzed by this enzyme together with lactic dehydrogenase. The

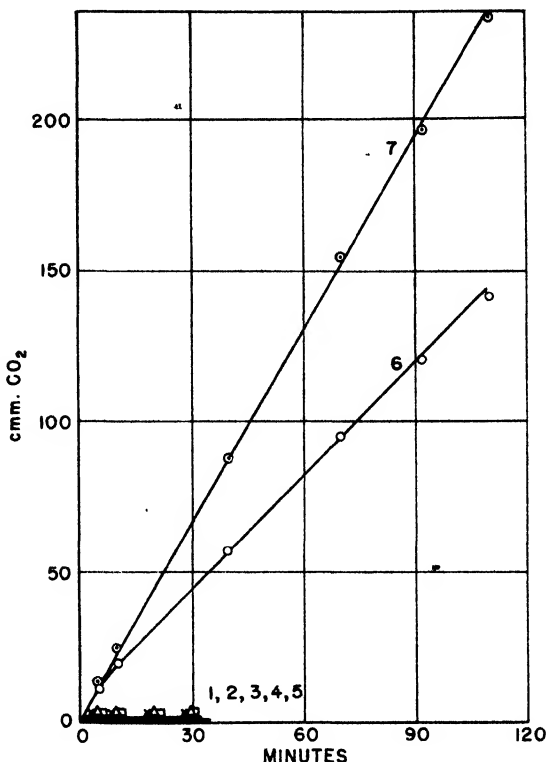


FIG. 3. Malate-pyruvate dismutation with purified pigeon liver enzyme. The complete system (Curve 7) contained 0.1 M acetate buffer, pH 5.2, 5 micromoles of  $\text{MnCl}_2$ , 15 micromoles of *l*-malate (336 cmm.), 60 micromoles of pyruvate, 0.135 micromole of TPN, excess of crystalline lactic dehydrogenase, and liver enzyme (specific activity 450) with 290  $\gamma$  of protein. Curve 6, without lactic dehydrogenase. There was no reaction if either liver enzyme or TPN was omitted, if fumarate was substituted for malate, or if DPN (0.21 micromole) was substituted for TPN, either without or with 5 micromoles of ATP (Curves 1 to 5). Final volume 2.1 cc. Gas, air; temperature,  $38^\circ$ . The reaction was started by tipping in substrates from the side bulbs of Warburg vessels after temperature equilibration.

experiment illustrated in Fig. 3 was carried out with the purest preparation available of the pigeon liver enzyme (specific activity 450; see Ochoa *et al.* (12)). This preparation still contains some lactic dehydrogenase but in amounts insufficient for a maximum rate of dismutation. Under these

conditions, the reaction is accelerated by addition of crystalline lactic dehydrogenase. Since the purified enzyme is free from fumarase and other enzymes originally present in pigeon liver extracts and crude ammonium sulfate fractions, undesirable side reactions are excluded. Thus, the reaction proceeds only in the presence of *l*-malate, pyruvate,  $Mn^{++}$ , and TPN. DPN, with or without ATP, has no effect, and fumarate is inert when substituted for malate.

A chemical balance of the malate-pyruvate dismutation, carried out with ammonium sulfate fractions of pigeon liver (Table II), is in complete agreement with Equation 1, as reported by Evans *et al.* (4). It will be observed that, with the crude enzyme used, fumarate reacted as well as malate and also that all of the dicarboxylic acid reacts if sufficient time is

TABLE II

*Chemical Balance of Dismutation between Malate and Pyruvate*

0.08 M acetate buffer, pH 5.2, 5 micromoles of  $MnCl_2$ , 0.135 micromole of TPN, 8.9 micromoles of pyruvate, and 1.0 cc. of pigeon liver enzyme (dialyzed ammonium sulfate fraction as in Fig. 2), with other additions as indicated. Final volume 2.5 cc. Gas, air; temperature, 38°. The reaction was started by tipping in fumarate or malate from the side bulbs of Warburg vessels after temperature equilibration. Incubation, 100 minutes. The results are expressed in micromoles.

Experiment No.	Additions	$\Delta CO_2$	$\Delta$ lactate	$\Delta$ pyruvate
1	Fumarate (13.4 micromoles)	+13.4	+13.4	-0.33
2	<i>l</i> -Malate (13.4 " )	+13.6	+11.8	-0.40
3	" (53.6 " )	+33.3	+28.2	-0.23*

\*Determined enzymatically with lactic dehydrogenase.

allowed. This fact, which had already been noted by Moulder *et al.* (5), has been made use of for a rapid and specific determination of malate (12).

*Isocitrate-Pyruvate Dismutation*—The occurrence of this reaction in pigeon liver extracts, as described by Moulder *et al.* (5), was confirmed. However, with dialyzed ammonium sulfate fractions, the reaction was found to be TPN-specific (Fig. 4). With extracts of washed, acetone-dried, pig heart (which contain large amounts of isocitric (and malic) dehydrogenase and oxalosuccinic carboxylase, but little or no aconitase or lactic dehydrogenase (13)) there is a rapid reaction as soon as a mixture of extract, isocitrate, pyruvate,  $Mn^{++}$ , and TPN is supplemented with crystalline lactic dehydrogenase; DPN cannot replace TPN (Fig. 4). Extracts of acetone-dried pig liver also catalyze the reaction with TPN as coenzyme. These results support the view that the isocitrate-pyruvate dismutation is catalyzed by isocitric dehydrogenase, oxalosuccinic carboxylase, and lactic

dehydrogenase, and is strictly TPN-specific. A chemical balance of this dismutation, as catalyzed by pig heart extract together with lactic dehy-

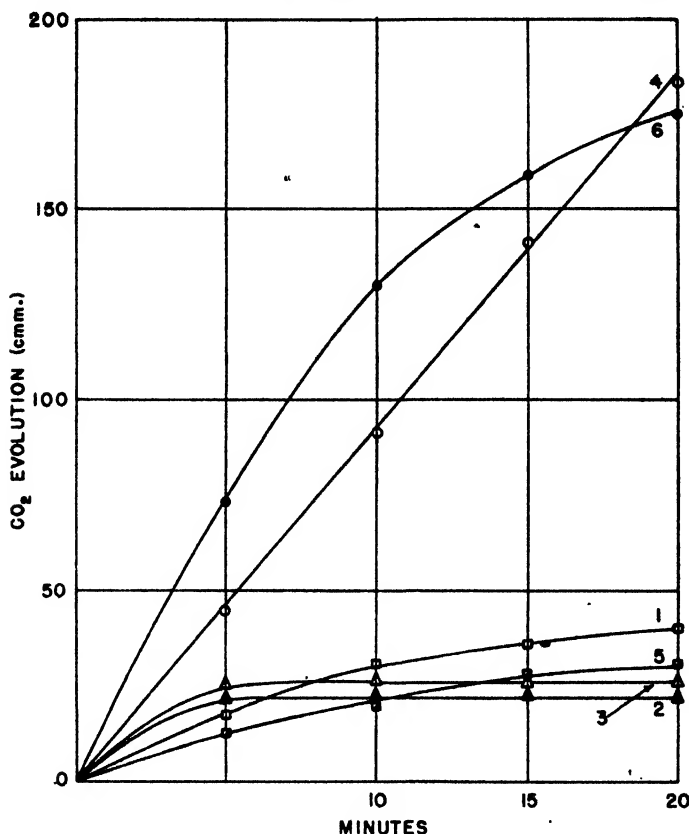


FIG. 4. Isocitrate-pyruvate dismutation. 0.08 M acetate buffer, pH 5.2, 5 micromoles of  $\text{MnCl}_2$ , 20 micromoles of *DL*-isocitrate (224 cmm.), 50 micromoles of pyruvate, and either 1.0 cc. of pig heart extract (Curves 1 to 4) or 0.5 cc. of dialyzed ammonium sulfate fraction of pigeon liver extract (fraction obtained between 0.33 and 0.56 saturation with ammonium sulfate; otherwise as that described in Fig. 2 except that the dialyzed solution was kept for 2 days at 3–4° before use) (Curves 5 and 6), with other additions as indicated. Final volume 2.5 cc. Gas, air; temperature, 38°. The reaction was started by tipping in substrates from the side bulbs of Warburg vessels after temperature equilibration. Curve 1, 0.07 micromole of TPN; Curve 2, crystalline lactic dehydrogenase; Curve 3, lactic dehydrogenase and 0.1 micromole of DPN; Curve 4, lactic dehydrogenase and 0.07 micromole of TPN; Curve 5, 0.1 micromole of DPN; Curve 6, 0.07 micromole of TPN.

drogenase, is shown in Table III and agrees with that obtained by Moulder *et al.* (5) with pigeon liver extracts.

TABLE III

*Chemical Balance of Dismutation between Isocitrate and Pyruvate*

0.085 M acetate buffer, pH 5.2, 10 micromoles of  $\text{MnCl}_2$ , 0.2 micromole of TPN, 42 micromoles of *dl*-isocitrate, 25 micromoles of pyruvate, 1.5 cc. of pig heart extract, and an excess of crystalline lactic dehydrogenase. Final volume 3.5 cc. Gas, air; temperature, 38°. The reaction was started by tipping in isocitrate from the side bulbs of Warburg vessels after temperature equilibration. Incubation, 60 minutes. The results are expressed in micromoles.

Method of estimation		Initial	Final	$\Delta$
Pyruvate	Hydrazone	25.1	11.0	-14.1
$\alpha$ -Ketoglutarate	Oxidation and succinoxidase	0.0	12.4	+12.4
"	Hydrazone	0.0	13.3	+13.3
$\text{CO}_2$	Manometric		13.1	+13.1
Lactate	Colorimetric	1.4	15.9	+14.5

TABLE IV

*Pyridine Nucleotide Specificity of Some Dehydrogenases*

Dehydrogenase	Source and purity	Reaction tested*	Reactive pyridine nucleotide
Malic	Pig heart (crude† or pure), pigeon liver‡	Oxalacetate + $\text{CoH}_2 \rightleftharpoons$ <i>l</i> -malate + Co	DPN, TPN
Lactic	Ox heart (crystalline), pigeon liver‡	Pyruvate + $\text{CoH}_2 \rightleftharpoons$ lactate + Co	DPN, TPN
Isocitric§	Pig heart,† pig liver,   pigeon liver‡ (all crude)	<i>d</i> -Isocitrate + Co $\rightleftharpoons$ $\alpha$ -ketoglutarate + $\text{CO}_2$ + $\text{CoH}_2$	TPN
Glutamic	Pig liver   (crude), calf liver (partially purified)	<i>L</i> -Glutamate + Co $\rightleftharpoons$ $\alpha$ -ketoglutarate + $\text{NH}_3$ + $\text{CoH}_2$	DPN, TPN
Triose phosphate¶	Rabbit muscle (crystalline)	3-Phosphoglyceraldehyde + Co $\rightarrow$ 3-phosphoglycerate + $\text{CoH}_2$	DPN

\* The direction in which individual reactions were tested is indicated by the heavy arrows. Co stands for coenzyme (pyridine nucleotide).

† Dialyzed phosphate extract of washed, acetone-dried, pig heart (8).

‡ Dialyzed aqueous extract of acetone-dried pigeon liver (4, 5).

§ In the presence of oxalosuccinic carboxylase and  $\text{Mn}^{++}$  (8).

|| Dialyzed phosphate extract of acetone-dried pig liver.

¶ In the presence of arsenate (17).

*Pyridine Nucleotide Specificity of Dehydrogenases*—A reinvestigation of the pyridine nucleotide specificity of several dehydrogenases was undertaken for the reasons already stated. In order to obtain unequivocal results, the rate of oxidation or reduction of the pyridine nucleotide, in the

presence of enzyme and substrate, was measured spectrophotometrically by the methods developed by Warburg (18). All of the reactions studied are reversible. They were followed in the direction in which they proceed at a faster rate, except in the case of the reaction catalyzed by glutamic dehydrogenase, which was followed in both directions. The results are summarized in Table IV.

TABLE V

*Reaction of Malic and Lactic Dehydrogenases with Pyridine Nucleotides*

Each experimental cell contained 0.025 M glycylglycine buffer, pH 7.4, enzyme and pyridine nucleotide as indicated, and water to a volume of 2.9 cc. At zero time 0.1 cc. of either 0.0076 M oxalacetate (malic dehydrogenase) or 0.0091 M pyruvate (lactic dehydrogenase) was added. Temperature, 22–24°. The blank cell contained no pyridine nucleotide. The reaction rates were calculated from the third 15 second period after addition of substrate.

Dehydro- genase	Source	Reduced pyridine nucleotide	Pyridine nucleotide concentration	Amount of enzyme	Rate	Activity ratio*
			<i>mole</i> $\times 10^{-4}$ <i>per cc.</i>	<i>cc.</i>	<i>mole</i> $\times 10^{-4}$ <i>per min.</i> <i>per cc.</i>	
Malic	Pig heart (pure)	DPN	5.3	0.0005	2.1	34
		TPN	11.0	0.005	0.61	
	" " extract	DPN	7.7	0.001	1.0	25
		TPN	7.5	0.02	0.81	
	Pigeon liver extract	DPN	5.7	0.0015	1.0	17
		TPN	5.7	0.01	1.2	
Lactic	Ox heart (crystalline)	DPN	5.6	0.0001	0.71	220
		TPN	7.3	0.02	0.64	
	Rabbit muscle†	DPN	4.2	0.0005	0.95	170
		TPN	7.4	0.1	1.1	
	Pigeon liver extract	DPN	6.3	0.001	1.35	135
		TPN	7.5	0.1	1.0	

\* Rate with DPN per cc. enzyme

Rate with TPN per cc. enzyme

† Crystalline fraction supplied by Dr. E. Racker.

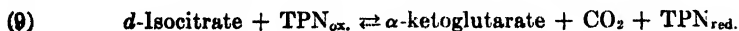
Whereas, in confirmation of von Euler *et al.* (16), the liver glutamic dehydrogenase was found to react about equally fast with either DPN or TPN, in the case of malic, and especially of lactic dehydrogenase, the reaction with DPN was much faster. This is illustrated in Table V.

#### DISCUSSION

The results presented in this paper make it possible to interpret the reactions between either malate or isocitrate and pyruvate as TPN-linked



dismutations resulting in the oxidative decarboxylation of malate or isocitrate, with simultaneous reduction of pyruvate to lactate. These reactions proceed according to Equations 8, 9, and 10, in which the main direction is indicated by heavy arrows. Reaction 8 is catalyzed by the new enzyme from pigeon liver, Reaction 9 by isocitric dehydrogenase together with oxalosuccinic carboxylase, and Reaction 10 by lactic dehydrogenase.



The TPN specificity of the above dismutations is due to the strict TPN specificity of Reactions 8 and 9. Reaction 10 proceeds much faster with DPN than with TPN; the fact that it occurs at all with TPN makes the dismutations possible. Under these conditions, Reaction 10 is probably the reaction limiting the over-all rate of the dismutations.

A faster dismutation should occur between either malate or isocitrate and oxalacetate with the malic dehydrogenase (instead of lactic dehydrogenase) system as oxidant, since malic dehydrogenase reacts with TPN relatively more rapidly than lactic dehydrogenase does. Still faster dismutations should occur, in the presence of  $\alpha$ -ketoglutarate and  $\text{NH}_3$ , with the glutamic dehydrogenase system as oxidant, since its high oxidation-reduction potential is as favorable as that of the lactate and malate systems, and its reaction with TPN is rapid. It will be recalled that Adler *et al.* (14) described such a reaction: a rapid conversion of isocitrate +  $\text{NH}_3$  to glutamate.

The failure of purified malic dehydrogenase, oxalacetic carboxylase, and lactic dehydrogenase to catalyze the malate-pyruvate dismutation simply reflects the failure of a combination of the first two enzymes to catalyze the oxidative decarboxylation of malic acid. The problem presented by this fact is discussed in the following paper (12).

### Methods

*Isolation of Oxalacetic Carboxylase from Micrococcus lysodeikticus*—The organisms were grown and harvested as described by Krampitz and Werkman (7). Suspensions of washed organisms were lysed with lysozyme. Lysozyme was prepared by the method of Alderton *et al.* (19), followed up to the lyophilization of the dialyzed eluate from bentonite. The lysed preparation was brought to pH 5.0 with 1.0 N acetic acid and the precipitate was centrifuged off and discarded. The supernatant was shaken with alumina gel C $\gamma$  (which adsorbs most of the flavoprotein present) and the

supernatant was precipitated with 3 volumes of acetone at 0°. The precipitate was collected by centrifugation, washed twice with ice-cold acetone, and dried *in vacuo*. 1.1 gm. of pooled acetone powder were ground in a mortar with 120 cc. of 0.1 M phosphate buffer, pH 7.4, the insoluble residue was centrifuged off, and the supernatant was dialyzed for 3 to 4 hours against running tap water. To the dialyzed solution were added, with stirring, 19.2 mg. of protamine sulfate (dissolved in 1.9 cc. of water and brought to pH 6.0), and the mixture was centrifuged at 3000 R.P.M. for 30 minutes. To 135 cc. of slightly turbid supernatant were added 23.8 gm. of ammonium sulfate, and the precipitate was discarded. The enzyme was precipitated by the further addition of 30 gm. of ammonium sulfate. The precipitate was dissolved in a small amount of water and the solution was dialyzed overnight against running tap water. The enzyme was now precipitated with 4 volumes of acetone at 0°. The precipitate was washed twice with ice-cold acetone and dried *in vacuo*. This preparation is very stable, retaining full activity for over a year when stored at 3–4°. It is free of lactic and malic dehydrogenases and quite inactive toward malic acid whether with DPN or TPN.

*Oxalacetic Carboxylase Test*—The manometric activity tests are based on the rate of decarboxylation of oxalacetate and are carried out in the presence of 0.1 M acetate buffer, pH 5.2, 1 micromole of  $MnCl_2$ , enzyme, and 19 micromoles (425 c.mm.) of freshly dissolved oxalacetate which is tipped in from the side bulb of the Warburg vessels after 5 minutes of temperature equilibration. Final volume 2.0 cc.; gas, air; temperature, 25°. A blank without enzyme is carried out simultaneously to correct for the spontaneous decarboxylation of oxalacetate. Under the above conditions the evolution of  $CO_2$ , in the presence of enzyme, is of zero order over a good part of its course. The spontaneous decarboxylation is first order. 1 enzyme unit is defined as the amount of enzyme causing an excess  $CO_2$  evolution over the blank of 1.0 c.mm. in 10 minutes, calculated for the second 5 minutes following tipping in the oxalacetate. The corrected  $CO_2$  evolution in this period is proportional to enzyme concentration within rather wide limits. The specific activity (units per mg. of protein) of the above preparation of oxalacetic carboxylase was about 200. The specific activity of the lysed cells was about 50.

*Dehydrogenase Preparations*—Pure malic dehydrogenase was prepared from pig heart according to Straub (9). Crystalline lactic dehydrogenase was prepared from ox heart by the method of Straub (10). Crystalline triose phosphate dehydrogenase, prepared from rabbit muscle by the method of Cori *et al.* (17), was kindly supplied by Dr. E. Racker. A preparation of glutamic dehydrogenase from acetone-dried calf liver, partially purified by fractionation with ethanol at low temperature, was kindly supplied by

Dr. S. Ratner. The nature of the various crude enzyme preparations used is indicated in Table IV.

*Dehydrogenase Tests and Units*—The rate of oxidation or reduction of the reduced or oxidized pyridine nucleotide was measured by determining the absorption of light of wave-length 340  $\mu$  in the Beckman spectrophotometer. The reactions thus measured in the case of each of the various dehydrogenases were those indicated in Table IV. The reaction mixtures containing 0.025 M glycylglycine buffer, pH 7.4, pyridine nucleotide, enzyme, substrate, water, and, if necessary, other additions, were made up to a final volume of 3.0 cc. in quartz cells of 1.0 cm. light path. Reactions were started by addition of either substrate or enzyme and readings of the optical density were taken, at intervals of 15 seconds, against a blank containing all components except pyridine nucleotide. 1 unit was arbitrarily defined, in every case, as the amount of enzyme causing a  $\Delta \log I_0/I$  of 0.01 per minute calculated for the third 15 second period after the start of the reaction. The concentrations of substrates and pyridine nucleotides were chosen to give maximum rates with the highest amounts of enzyme used, which did not exceed 10 units. The tests were carried out at room temperature between 20–25°.

*Chemical Preparations*—The following substances were prepared as previously described: crystalline sodium pyruvate (20), DPN<sup>2</sup> (21), *dl*-isocitric acid (8), oxalacetic acid (22). Fumaric and *l*-malic acids were obtained commercially. Reduced DPN (sodium salt) was prepared by the method of Ohlmeyer (23). Solutions of reduced TPN were obtained by enzymatic reduction with stoichiometric amounts of isocitric acid. After completion of the reaction, the mixture was brought to about pH 10.5 with dilute alkali, heated for 2 to 3 minutes at 100°, cooled, and centrifuged.

The DPN preparations were free from TPN as shown by their failure to catalyze the reduction of 2,6-dichlorophenol indophenol by glucose-6-phosphate in the presence of *Zwischenferment* (24). The absence of DPN from the TPN preparations was shown by their inactivity in the triose phosphate dehydrogenase system.

*Analytical Methods*—Pyruvic and  $\alpha$ -ketoglutaric acids were determined by the method of Friedemann and Haugen (25) and lactic acid by the method of Barker and Summerson (26). Pyruvic acid was also determined enzymatically with lactic dehydrogenase (12).

*Enzymatic Determination of TPN*—TPN was determined by its catalytic effect on the rate of reduction of 2,6-dichlorophenol indophenol by isocitric acid, in the presence of isocitric dehydrogenase, according to the method of Haas (24). The test system was made up with enzyme, 0.3 cc. of 0.25

<sup>2</sup> We are greatly indebted to Dr. Charles N. Frey and Mr. R. F. Light, The Fleischman Laboratories, New York, for generous supplies of yeast for this preparation.

M glycylglycine buffer, pH 7.4, 0.02 cc. of 0.05 M  $\text{MnCl}_2$ , 0.2 cc. of 2,6-dichlorophenol indophenol (0.1 mg. per cc.), 0.1 mg. of *dl*-isocitrate, TPN or the TPN-containing sample, and water to a volume of 3.0 cc. Readings were taken at room temperature (20–22°) in the Beckman spectrophotometer at wave-length 600  $\text{m}\mu$ . The blank cell contained no dye. The reaction was started by the addition of isocitrate or enzyme. The early rate of dye reduction is, within certain limits, proportional to the TPN concentration, as is shown in Fig. 5. There is some blank reaction if dialyzed heart extracts are used; this can be eliminated by the use of partially purified preparations (8).

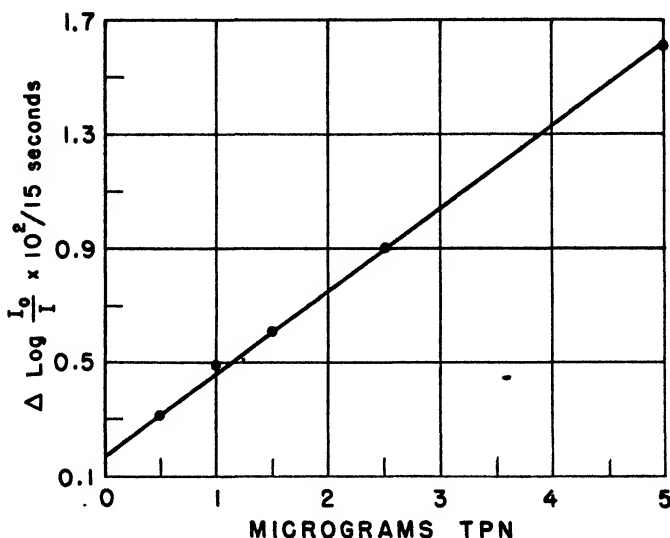


FIG. 5. Rate of reduction of 2,6-dichlorophenol indophenol by the isocitric dehydrogenase system as a function of the TPN concentration. Wave-length, 600  $\text{m}\mu$ .

Reaction mixtures were diluted with 0.5 volume of water and transferred to small test-tubes. These were placed in boiling water for 1 minute, cooled in ice, and centrifuged at high speed. The clear supernatants were analyzed for TPN as described above.

#### SUMMARY

The reaction between malate and pyruvate, yielding pyruvate,  $\text{CO}_2$ , and lactate, specifically requires TPN as coenzyme. This reaction is a TPN-linked dismutation involving both an enzyme isolated from pigeon liver, catalyzing the oxidative decarboxylation of malate to pyruvate and  $\text{CO}_2$ , and lactic dehydrogenase. A mixture of purified malic dehydrogenase,

oxalacetic carboxylase, and lactic dehydrogenase failed to catalyze this reaction.

The reaction between isocitrate and pyruvate, yielding  $\alpha$ -ketoglutarate,  $\text{CO}_2$ , and lactate, is TPN-specific and proceeds in the presence of dialyzed extracts of washed, acetone-dried, pig heart (containing isocitric dehydrogenase and oxalosuccinic carboxylase) supplemented with crystalline lactic dehydrogenase. This reaction is also a TPN-linked dismutation involving both the isocitric and lactic dehydrogenase systems.

A reinvestigation of the pyridine nucleotide specificity of several dehydrogenases, by direct spectrophotometric measurement of the oxidation or reduction of the nucleotide, showed that malic and lactic dehydrogenase can react with either DPN or TPN, but the reaction with the latter is slower. The coenzyme specificity of other dehydrogenases (isocitric, triose phosphate, glutamic) conforms to that found by earlier observers.

The TPN specificity of the malate-pyruvate and isocitrate-pyruvate dismutations is determined by the TPN specificity of the reductant systems, since the oxidant system, lactic dehydrogenase, can react with either DPN or TPN and indeed reacts much faster with the former.

In crude enzyme preparations of pigeon liver there is a slow synthesis of TPN from DPN and ATP. Such preparations also bring about a slow disappearance of added TPN.

We wish to thank Mr. Sidney Udenfriend for help with some of the experiments, and Mr. Morton C. Schneider for technical assistance.

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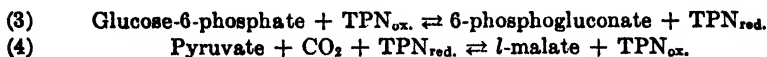
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system of low oxidation-reduction potential. The following reactions are then obtained.



The net result of Reactions 3 and 4 is Reaction 5, a TPN-linked dismutation whereby glucose-6-phosphate is oxidized to phosphogluconate, while pyruvate is carboxylated and reduced to malate.



#### EXPERIMENTAL

*Optical Test and Enzyme Unit*—Activity determinations are based on Reaction 1. The early rate of reduction of  $\text{TPN}_{\text{ox.}}$  in the presence of enzyme, manganous ions, and an excess of *l*-malate is, within certain limits, proportional to the enzyme concentration. The formation of  $\text{TPN}_{\text{red.}}$  is followed in the Beckman spectrophotometer at wave-length 340  $\text{m}\mu$ . Measurements have been carried out at room temperature (20–24°).

The reaction mixture, in quartz cells ( $d = 1.0 \text{ cm.}$ ), consists of 0.025  $\text{M}$  glycylglycine buffer, pH 7.4, 1.0 micromole of  $\text{MnCl}_2$ , 0.135 micromole of  $\text{TPN}_{\text{ox.}}$ , 0.448 micromole of *l*-malate, enzyme, and water. Final volume 3.0 cc. The reaction is started by the addition of malate, and readings of the optical density are made, against a blank containing all components except TPN, at intervals of 15 seconds for 1 or 2 minutes. The increase in optical density ( $\Delta \log I_0/I$ ) between 30 and 45 seconds after the addition of malate is used to calculate the enzyme activity. 1 enzyme unit has been arbitrarily defined as the amount of enzyme causing an increase in optical density of 0.01 per minute. The amount of enzyme used in a test is adjusted so that  $\Delta \log I_0/I$ , for the period between 30 and 45 seconds, lies between +0.006 and +0.015.

The protein content of enzyme fractions is determined spectrophotometrically by measuring the absorption of light at wave-lengths 280 and 260  $\text{m}\mu$ . The protein concentration was calculated, from the absorption at 280  $\text{m}\mu$ , with a correction for the nucleic acid content from the data given by Warburg and Christian (4). The specific activity of an enzyme preparation is given by the ratio, units per  $\text{mg.}$  of protein.

#### *Isolation of Enzyme*

The starting material is acetone powder of pigeon liver. Preparation of the powder and extraction are carried out essentially according to Evans *et al.* (5). The enzyme has been substantially purified by fractionation with ethanol at low temperature and fractional adsorption on alumina gel.

*Preparation of Acetone Powder*—Pigeon livers, removed as rapidly as possible after decapitation, were cooled in ice and freed from fat and connective tissue. The livers were extracted with acetone, cooled to  $-5^{\circ}$ , in a chilled Waring blender; the suspension was then poured into sufficient acetone (at  $-5^{\circ}$ ) to make a total of 10 volumes of acetone. The mixture was stirred for a few minutes and filtered with suction in the cold room. The residue was reextracted once more with acetone, spread on filter paper, and allowed to dry at room temperature. The dry material was freed from the larger threads of connective tissue, ground to a powder in a mechanical mortar, and stored in stoppered bottles in the ice box. Its activity keeps unchanged for several months.

*Step 1. Extraction*—100 gm. of acetone powder were extracted for 10 minutes with 1 liter of distilled water at  $40^{\circ}$  with mechanical stirring; the insoluble residue was centrifuged off at room temperature and discarded. This yielded 800 to 900 cc. of a reddish brown turbid extract, the protein concentration of which was about 25 to 30 mg. per cc. Its specific activity was about 4.

*Step 2. First Ethanol Fractionation*—To 800 cc. of extract were added 200 cc. of 0.2 M phosphate buffer, pH 7.4, after cooling both to  $0^{\circ}$ . Absolute alcohol (cooled to  $-30^{\circ}$ ) was now added to the solution with mechanical stirring. The alcohol was added very slowly and the temperature was not allowed to rise above  $0^{\circ}$ . As the alcohol concentration rises, the temperature<sup>1</sup> is allowed to fall eventually to  $-5^{\circ}$  and is maintained until enough alcohol has been added to make a concentration of 32 to 35 per cent by volume.

The precipitate was centrifuged off at  $-5^{\circ}$ , in the refrigerated centrifuge, and discarded. The supernatant was allowed to stand overnight at  $-15^{\circ}$ , whereby a further precipitate was obtained. This precipitate was centrifuged off at  $-10^{\circ}$  and dissolved in ice-cold 0.02 M phosphate buffer, pH 7.4. It gave a clear red solution, specific activity 13. Solutions from three 100 gm. batches of acetone powder, each obtained as described above, were combined at this stage to give 194 cc. of solution (see Table V) containing 55 mg. of protein per cc.

*Step 3. Refractionation with Ethanol*—On fractionation of 194 cc. of the above solution the results shown in Table I were obtained.

Fractions obtained by precipitation with ethanol at a given temperature were always collected by centrifugation at the same temperature and taken up in ice-cold 0.02 M phosphate buffer, pH 7.4. While the first fraction of any series usually contained some insoluble material, subsequent fractions always gave clear solutions.

<sup>1</sup> The desired temperatures were maintained with alcohol-water mixtures which were kept as semifrozen slushes by means of an outer bath of alcohol (or acetone) and dry ice.

Fractions 1 and 3 were combined and refractionated to yield Fraction 4, containing 21,800 units of specific activity 25.0.

Fractions 2 and 4 were combined; the solution was dialyzed overnight against 0.1 M phosphate buffer, pH 7.4, and diluted with an equal volume

TABLE I  
*Second Ethanol Fractionation*

Fraction No.	Ethanol added to supernatant	Temperature	Units in solution of ppt.	Specific activity
	cc.	°C.		
1	14	-1	59,000	11.7
2	9	-5	84,500	23.4
3	9	-5	6,480	5.7

TABLE II  
*Refractionation with Ethanol*

Fraction No.	Ethanol added to supernatant	Temperature	Units in solution of ppt.	Specific activity
	cc.	°C.		
1a	40	-5	4,060	6.9
2a	10	-5	20,700	13.2
3a	10	-5	23,000	23.3
4a	10	-5	38,000	36.8
5a	0	-15	6,400	9.5

TABLE III  
*Refractionation with Ethanol*

Fraction No.	Ethanol added to supernatant	Temperature	Units in solution of ppt.	Specific activity
	cc.	°C.		
1b	5	-5	3,360	12.4
2b	1	-5	12,400	16.4
3b	5	-5	26,600	48.0
4b	0	-10	11,800	45.5
5b	5	-8	1,850	29.0

of water to give 200 cc. of solution containing 23 mg. of protein per cc. This solution was fractionated with ethanol; the results are shown in Table II.

Fractions 2a and 3a were combined, diluted to 60 cc. with 0.02 M phosphate buffer, pH 7.4 (protein concentration about 40 mg. per cc.), and fractionated (see Table III).

Fractions 4a, 3b, and 4b were combined to give 30 cc. of solution containing 72,700 units, specific activity 43.0, with 55.5 mg. of protein per cc. (Table V).

*Step 4. Fractional Adsorption on Alumina Gel*—30 cc. of solution from Step 3 were cooled to 0° and diluted with 119 cc. of ice-cold water. To this solution were added slowly, with mechanical stirring, 15 cc. of ice-cold 2.0 M acetate buffer, pH 4.8. This gives 164 cc. of a clear red solution containing about 10 mg. of protein per cc., pH 5.0. To this solution were added 11.0 cc. of a suspension of alumina gel C $\gamma$  (after Willstätter), containing 14.5 mg. of Al<sub>2</sub>O<sub>3</sub> per cc., with mechanical stirring. Stirring was continued for 10 minutes, while the temperature of the mixture was kept at 0°, and the alumina was centrifuged off in the cold room. To the supernatant were added a further 11.0 cc. of alumina suspension and the mixture was worked up as before. This was repeated three more times. Each alumina residue was separately eluted at 0° with 9.0 cc. of 0.1 M phosphate buffer, pH

TABLE IV  
*Analysis of Eluates from Alumina Gel (Step 4)*

Eluate No.	Units	Specific activity
1	3,640	31.7
2	6,250	58.5
3	15,800	178.0
4 (Table V)	21,100	272.0
5	2,160	62.0
3a	2,820	100.0
4a	3,920	171.0

7.4. Residues 3 and 4 were reeluted with an additional 5.0 cc. of phosphate buffer each (eluates 3a and 4a). Analysis of the various eluates gave the results presented in Table IV.

Eluate 4 was a clear, pale yellow, solution; it contained 6 mg. of protein per cc.

*Step 5. Refractionation with Ethanol and Alumina Gel*—Eluates 3, 3a, and 4a were combined and refractionated with ethanol. A fraction was obtained containing 12,000 units, specific activity 190. This solution (3.0 cc.) was brought to pH 4.9 with 0.7 cc. of 2.0 M acetate buffer, pH 4.8, at 0°. The clear solution thus obtained, containing 17.0 mg. of protein per cc., was fractionated at 0° as before by successive adsorptions on alumina gel. Additions of 0.3, 0.3, 0.3, and 0.6 cc. of the alumina suspension were made. Each residue was eluted with 2.0 cc. of 0.1 M phosphate buffer, pH 7.4. Residue 4 was eluted once more with 1.0 cc. of buffer (eluate 4A<sub>1</sub>). The following results were obtained.

Eluate No.	Units	Specific activity
2A	1520	206
3A	1920	275
4A	3200	402
4A <sub>1</sub>	468	287

Fractions of specific activity between 200 and 300 (*i.e.*, eluates 4, 2A, 3A, and 4A<sub>1</sub>) were combined and refractionated with alumina gel. The best fraction thus obtained contained 8640 units, specific activity 450 (Table V). This was a clear, very faintly yellowish solution containing 4.8 mg. of protein per cc.

TABLE V  
*Purification of Pigeon Liver Enzyme*

300 gm. of acetone powder.

Step	Vol- ume of solu- tion	Units	Protein	Specific activity	Yield	Over- all re- covery in step
	cc.		mg.	units per mg. protein	per cent	per cent
Aqueous extract*	2470	276,500	64,500	4	100	
1st ethanol fractionation	194	155,000	11,600	13	56	90-100
Refractionation with ethanol	30	72,700	1,690	43	26	90-100
Elution from alumina gel	13	21,000	77.5	272	7.6	80
Refractionation with etl nol and alumina gel	4	8,640	19.2	450	3.2	80

\* Small aliquot dialyzed for tests.

Table V summarizes the pertinent data on the various purification steps. The last column gives the over-all recovery of enzyme for individual steps, obtained by adding up the yields from all the fractions in each. The high recoveries indicate that, in spite of extensive fractionation, little or no activity is being lost, as would most probably have been the case if more than one enzyme were involved in the catalysis of Reaction 1.

The enzyme is relatively stable, especially in the presence of phosphate. Solutions in 0.1 M phosphate buffer, pH 7.4, lose activity only very gradually if kept at 0°. Fractions of specific activity between 15 and 50 have been dialyzed against 0.1 M phosphate buffer, pH 7.4, for periods over 2 weeks, with little or no loss of activity.

While it is not yet possible to estimate the degree of purity attained, some of the enzymes present in large amounts in the pigeon liver extracts, such as malic and isocitric dehydrogenase, are almost completely removed by

the purification procedure described. Fumarase is completely removed early in the purification (1). Removal of lactic dehydrogenase, on the other hand, is as yet incomplete. These facts are illustrated in Table VI which shows the specific activities of several enzymes at various stages of purification of the pigeon liver enzyme. The activity of this enzyme as determined spectrophotometrically (*i.e.*, catalysis of Reaction 1) is referred to as "malic" enzyme activity and given in Column 2 of Table VI.

TABLE VI

Activity of Various Enzymes at Different Purification Stages of Pigeon Liver Enzyme

Fraction  (1)	Specific activity					Ratios					
	"Malic" enzyme (E) (2)	Oxalacetic carboxylase (O)		Lactic dehydro- genase (L) (5)	Malic dehydro- genase (M) (6)	Iso- citric dehydro- genase (I) (7)	O E (8)	O (TPN) E (9)	L E (10)	M E (11)	I E (12)
		With- out TPN (3)	With TPN* (4)								
Aqueous extract†	5.2	5.7		81	119	21	1.1		15.6	22.8	4.0
1st ethanol frac- tionation	13.1	9.7		71			0.74		5.4		
Ethanol fraction	21.0	11.7	24.9				0.59	1.18			
“ refrac- tionated	35.3	15.3	37.3				0.43	1.06			
“ “	46.5	23.2	49.4				0.50	1.06			
Alumina eluate	56.0	43.2		128			0.77		2.3		
“ “	220.0	93.0	218				0.42	1.00			
“ “	274.0	224.0		196	196	27	0.82		0.72	0.72	
Ethanol and alumi- na refractionated	350.0	243		415	83‡	14	0.70		1.2	0.24	0.04

\* 0.135 micromole.

† Data for "malic" enzyme, average of five estimations on different (dialyzed) extracts. Data for oxalacetic carboxylase and lactic dehydrogenase, average of three estimations; for isocitric dehydrogenase, of two estimations.

‡ May be apparent malic dehydrogenase activity since pyruvate, formed by decarboxylation of the oxalacetate used as substrate in this test, will oxidize DPN<sub>red</sub> owing to the presence of lactic dehydrogenase.

Tests and units of the other enzyme activities were as described in the preceding paper (2). Lactic and malic dehydrogenase activities were determined with reduced DPN, isocitric dehydrogenase activity with oxidized TPN. Columns 8 to 12 of Table VI give the ratios of the activity of each of the enzymes tested to "malic" enzyme activity.

Of special interest is the behavior of the oxalacetic carboxylase activity which, as first demonstrated by Evans *et al.* (5), is exhibited by pigeon liver

extracts. It will be observed, on inspection of Column 3 (Table VI), that the specific oxalacetic carboxylase activity is markedly increased on purification of the pigeon liver enzyme.

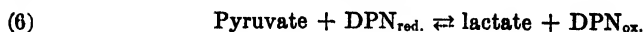
Vennesland, Evans, and Altman (6) made the remarkable observation that the rate of decarboxylation of oxalacetate by pigeon liver preparations is greatly stimulated by TPN, while DPN is inactive.<sup>2</sup> This observation has been fully confirmed in our laboratory. We have also observed that the effect of  $\text{TPN}_{\text{ox}}$  disappears after a short heating period at  $100^\circ$  in the presence of dilute alkali, while that of  $\text{TPN}_{\text{red}}$  is retained. It is also of interest that the rate of decarboxylation of oxalacetate by the purified carboxylase of *Micrococcus lysodeikticus* previously described (2) is not affected by TPN.

It may be observed by comparison of Columns 2, 3, and 4 of Table VI that, when the test for oxalacetic carboxylase is carried out in the presence of TPN, its specific activity increases on purification exactly to the same extent as does the "malic" enzyme activity. Column 9 (Table VI) shows that, under these conditions, the ratio of specific activities of oxalacetic carboxylase to "malic" enzyme remains constant over a 40-fold increase in purity. Unfortunately, we do not yet have oxalacetic carboxylase tests in the presence of TPN for fractions of specific activity higher than 220, but, from the increase in carboxylase activity produced by TPN on the various fractions investigated, a constant ratio of carboxylase to "malic" enzyme may be expected over the approximately 100-fold purification of the pigeon liver enzyme so far accomplished.

The above results strongly suggest that the same enzyme is responsible for catalysis of Reactions 1 and 2. Final decision on this point must, of course, await further purification.

### *Properties of Enzyme System*

*Formation of Pyruvate*—The formation of pyruvic acid from *l*-malic acid according to Reaction 1 has been demonstrated by means of lactic dehydrogenase. In the presence of lactic dehydrogenase and reduced DPN, pyruvic acid reacts according to Equation 6.



Since the equilibrium of Reaction 6 is very far to the right ( $K = (\text{pyruvate})(\text{DPN}_{\text{red}})/(\text{lactate})(\text{DPN}_{\text{ox}}) = 6 \times 10^{-5}$ , at pH 7.4 and  $22^\circ$  (7)), on addition of crystalline lactic dehydrogenase to a mixture of pyruvate and  $\text{DPN}_{\text{red}}$ , in which the latter is present in excess, practically all of the pyruvate is reduced to lactate, while an equivalent amount of  $\text{DPN}_{\text{red}}$  is oxidized with a corresponding decrease in the absorption of light at 340  $\mu$ . The

<sup>2</sup> We are indebted to Dr. E. A. Evans, Jr., and Dr. Birgit Vennesland for an early personal communication of this observation.

specificity of lactic dehydrogenase (8) makes this a rapid, sensitive, and specific method for the determination of pyruvate.

A typical experiment was carried out as follows: 1.34 micromoles of *l*-malate, 1.0 micromole of  $\text{MnCl}_2$ , pigeon liver enzyme (specific activity 100) with 0.5 mg. of protein, and glycylglycine buffer, pH 7.4, final concentration 0.025 M, were mixed at time zero with 0.74 micromole of  $\text{TPN}_{\text{ox}}$  in a quartz cell ( $d = 0.5$  cm.). Final volume 2.0 cc.; temperature,  $22^\circ$ . After 8 minutes, when the reaction markedly slowed down, the increase in optical density ( $\Delta \log I_0/I$ ) at  $340 \text{ m}\mu$  was  $+0.573$ , corresponding to the formation of 0.41 micromole of  $\text{TPN}_{\text{red}}$ .<sup>3</sup> At this time, 1.8 cc. of the reaction mixture were deproteinized with trichloroacetic acid, centrifuged, and a 0.5 cc. aliquot of the neutralized clear supernatant was mixed with 0.2 micromole of  $\text{DPN}_{\text{red}}$  and crystalline lactic dehydrogenase in 0.025 M glycylglycine buffer, pH 7.4. Final volume, 3 cc.;  $d = 1.0$  cm. The amount of  $\text{DPN}_{\text{red}}$  oxidized ( $\Delta \log I_0/I = -0.156$ ) was 0.083 micromole, which, on multiplication by the dilution factor (5.32), gives 0.44 micromole of pyruvic acid in the original 2.0 cc. sample.

*Components*—It has already been reported (1) that fumarate cannot be substituted for *l*-malate nor DPN for TPN ((1), Fig. 1, Curve 2). The same is true of *d*-malate. Phospho(enol)pyruvate cannot replace pyruvate. Further proof of the early removal of fumarase on purification of the pigeon liver enzyme is given in Fig. 1. Owing to the presence of fumarase in the pigeon liver extracts and some ammonium sulfate fractions thereof, they catalyze Reaction 1 equally well with either *l*-malate or fumarate as substrate. However, after ethanol and alumina fractionation, even fractions of low specific activity give almost no reaction with fumarate unless fumarase is added (Fig. 1, Curve 1).

Neither inorganic phosphate nor ATP participates in Reaction 1. In order to remove inorganic phosphate as far as possible, fractions of pigeon liver enzyme were repeatedly precipitated with ammonium sulfate from aqueous solution. With such fractions, a reaction mixture containing less than  $10^{-6}$  M phosphate gave rates of reduction of  $\text{TPN}_{\text{ox}}$  by malate that were not affected by addition of inorganic phosphate. Similarly, addition of ATP was without effect.

The need for manganous ions is illustrated in Fig. 1 (Curves 2 and 3); the rate of reaction is markedly slowed down in the absence of added  $\text{Mn}^{++}$ .

The pigeon liver enzyme cannot be replaced by a mixture of malic dehydrogenase (10) and oxalacetic carboxylase of *Micrococcus lysodeikticus* (2), whether with DPN or TPN. This is illustrated in Fig. 1 (Curve 4). The small increase in optical density following addition of  $\text{DPN}_{\text{ox}}$  (Curve 4,

<sup>3</sup> The molecular absorption coefficient of  $\text{TPN}_{\text{red}}$  at  $340 \text{ m}\mu$  ( $\epsilon = \ln(I_0/I)/c \times d$ ) was taken as  $1.3 \times 10^7$  (sq. cm. per mole) (9).



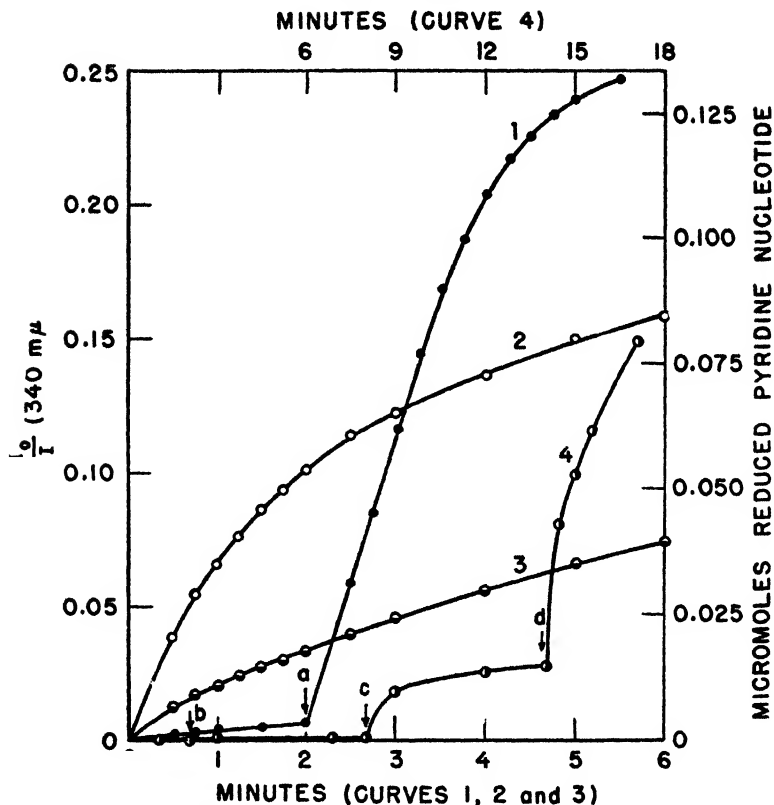


FIG. 1. Spectrophotometric tests with the liver enzyme. 0.025 M glycylglycine buffer, pH 7.4, with other additions as indicated. Final volume 3.0 cc.; temperature, 22–23°. Quartz cells;  $d = 1.0$  cm. The blank cells contained no TPN. Curve 1, 0.135 micromole of  $\text{TPN}_{\text{ox}}$ , 1.0 micromole of  $\text{MnCl}_2$ , liver enzyme (specific activity 75) with 400  $\gamma$  of protein and, at time zero, 0.448 micromole of fumarate; at Arrow *a* 0.01 cc. of fumarase (15) added. Curve 2, 0.135 micromole of  $\text{TPN}_{\text{ox}}$ , 1.0 micromole of  $\text{MnCl}_2$ , liver enzyme (specific activity 100) with 156  $\gamma$  of protein and, at time zero, 0.149 micromole of *l*-malate. Curve 3, same as Curve 2 but without  $\text{MnCl}_2$ . Curve 4, 1 micromole of  $\text{MnCl}_2$ , 0.3 mg. of oxalacetic carboxylase (60 units) from *Micrococcus lysodeikticus* (specific activity 200), 4.5 micromoles of *l*-malate and, at time zero, 0.135 micromole of  $\text{TPN}_{\text{ox}}$ ; at Arrow *b*, addition of purified malic dehydrogenase (1280 units); at Arrow *c*, addition of 0.210 micromole of  $\text{DPN}_{\text{ox}}$ ; at Arrow *d*, addition of liver enzyme (specific activity 168) with 44  $\gamma$  of protein (containing 7.5 units of oxalacetic carboxylase activity).

Arrow *c*) corresponds to the establishment of the equilibrium of the malate-oxalacetate system, catalyzed by malic dehydrogenase (Reaction 7), which lies very far in the direction of oxalacetate reduction.

(7)



The striking contrast with the action of the pigeon liver enzyme is shown by the effect of the addition of 7.5 units of this enzyme at Arrow *d* (Fig. 1, Curve 4). Negative results were also obtained, under similar conditions, with  $\text{DPN}_{\text{red.}}$ , pyruvate, and  $\text{CO}_2$  as initial reactants, whether in the absence or presence of ATP.

**Reversibility**—Proof of the reversibility of Reaction 1 has already been presented (1). The experiments were carried out by reducing  $\text{TPN}_{\text{ox.}}$  with malate in the presence of the enzyme (specific activity 168), following which the  $\text{TPN}_{\text{red.}}$  formed was oxidized on addition of pyruvate and a solution of sodium bicarbonate saturated with  $\text{CO}_2$ . As already pointed out (1), the presence of lactic dehydrogenase interferes by catalyzing the oxidation of  $\text{TPN}_{\text{red.}}$  by pyruvic acid (2). It is for this reason that the pigeon liver enzyme had to be largely freed from lactic dehydrogenase by purification before an unequivocal demonstration of the reversibility of Reaction 1 was possible.

Fig. 2 illustrates an experiment carried out with the purest fraction so far obtained (specific activity 450). Curve 1 shows the spectrophotometric course of the forward reaction with an amount of enzyme containing 14.5  $\gamma$  of protein in 3.0 cc. of reaction mixture; *i.e.*, less than 5  $\gamma$  per cc. Curves 2 and 3 show the course of the reverse reaction with twice as much enzyme. For the latter experiments  $\text{TPN}_{\text{red.}}$  was separately prepared by enzymatic reduction, as previously described (2), and was present in the reaction mixture from the beginning. It will be observed (Fig. 2, Curve 2) that, since some lactic dehydrogenase is still present, addition of pyruvate at time zero causes a slow oxidation of  $\text{TPN}_{\text{red.}}$ . However, the rate of oxidation thus obtained contrasts sharply with the very rapid rate that sets in on the further addition (at Arrow *a*) of  $\text{NaHCO}_3\text{-CO}_2$ , or with that obtained, on addition of pyruvate, when the  $\text{NaHCO}_3\text{-CO}_2$  solution was already present in the reaction mixture (Curve 3).

As already shown (1), the equilibrium of Reaction 1 must lie very far to the right, because if *l*-malate is present in excess with relation to  $\text{TPN}_{\text{ox.}}$ , practically all of the latter is reduced ((1), Fig. 1, Curve 1), while if, on the other hand,  $\text{TPN}_{\text{ox.}}$  is present in excess with relation to *l*-malate, practically all of the malate reacts to form pyruvate and  $\text{CO}_2$  ((1), Fig. 1, Curve 3).<sup>4</sup> The equilibrium constant of Reaction 1 cannot be determined until the enzyme is completely freed from lactic dehydrogenase.

**Decarboxylation of Oxalacetate**—The oxalacetic carboxylase activity of the purest pigeon liver enzyme preparation (specific activity 450) is shown in Fig. 3 (Curves 2 and 3). Curves 4 and 5, obtained with a preparation of

<sup>4</sup> In the legend to Fig. 1 of the paper quoted (1), the amounts of various components of reaction mixtures are erroneously expressed in millimoles (mm); the amounts should be in micromoles.

lower specific activity, illustrate the effect of manganous ions on this reaction.

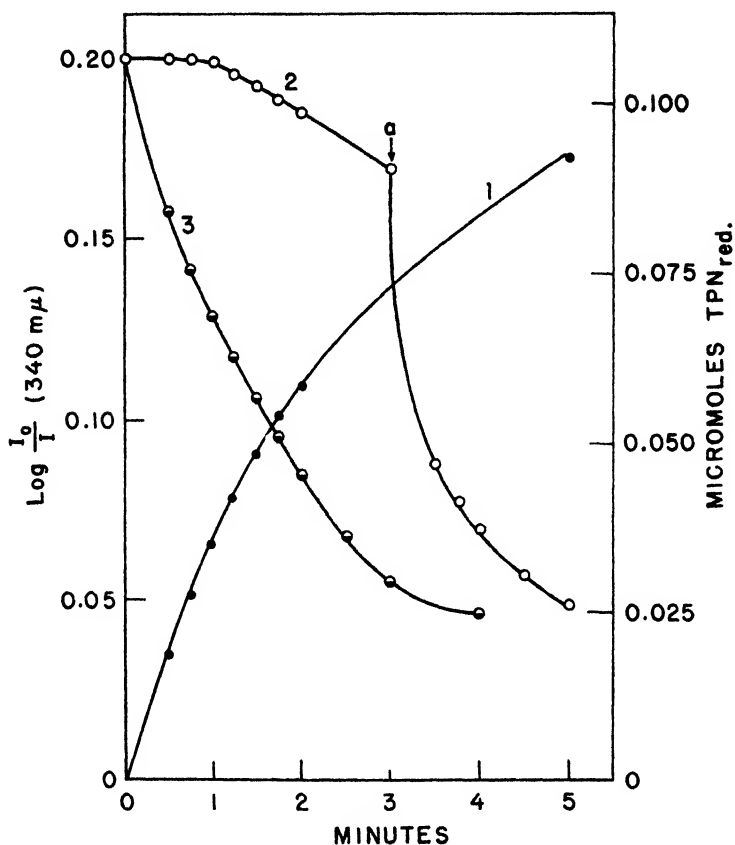


FIG. 2. Spectrophotometric tests with the liver enzyme; reversibility of Reaction 1. 0.025 M glycylglycine buffer, pH 7.4, and 1.0 micromole of  $\text{MnCl}_2$  with other additions as indicated. Final volume 3.0 cc.; temperature, 22°. Quartz cells;  $d = 1.0$  cm. The blank cells contained no TPN. Curve 1, 0.135 micromole of  $\text{TPN}_{\text{ox.}}$ , liver enzyme (specific activity 450) with 14.5  $\gamma$  of protein and, at time zero, 0.448 micromole of *l*-malate. Curves 2 and 3, 0.106 micromole of  $\text{TPN}_{\text{red.}}$ , liver enzyme (specific activity 450) with 29  $\gamma$  of protein, and, at time zero, 12.0 micromoles of pyruvate. Curve 2, 0.30 cc. of 0.1 M  $\text{NaHCO}_3$  (saturated with  $\text{CO}_2$  at room temperature) added at Arrow *a*; optical density readings corrected for dilution. Curve 3, 0.30 cc. of 0.1 M  $\text{NaHCO}_3$  saturated with  $\text{CO}_2$  was present from the beginning. The solutions used for these experiments were freshly prepared with  $\text{CO}_2$ -free water.

*Dismutation between Glucose-6-phosphate and Pyruvate +  $\text{CO}_2$* —Although, as stated above, the equilibrium of Reaction 1 lies very far in the direction

of decarboxylation, it can be shifted in the opposite direction by coupling with the glucose-6-phosphate dehydrogenase system, when Reaction 5

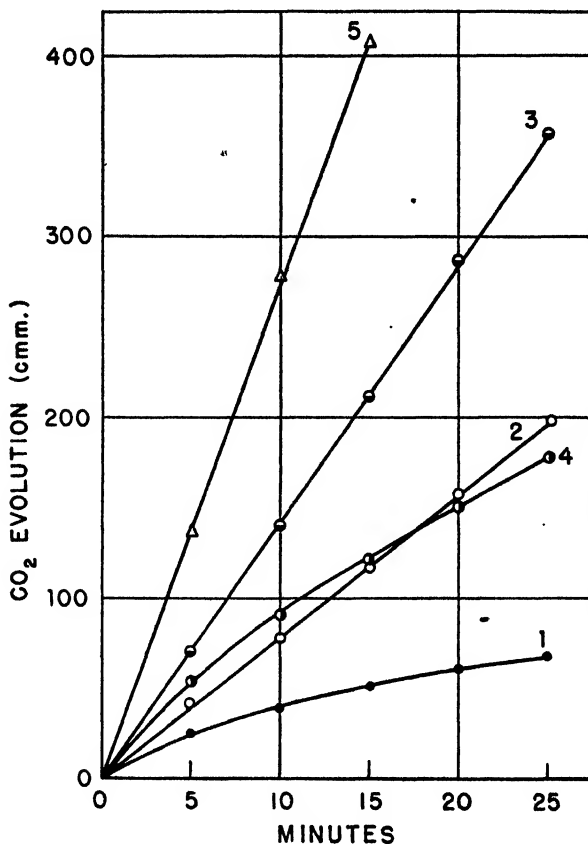


FIG. 3. Oxalacetic carboxylase activity of liver enzyme. 0.1 M acetate buffer, pH 5.2, and 19 micromoles (425 c.mm.) of oxalacetate with other additions as indicated. Final volume 2.0 cc. Gas, air; temperature, 25°. The reaction was started by tipping in oxalacetate (0.5 cc. of a freshly prepared solution containing 5.0 mg. per cc., pH 5.2) from the side bulbs of Warburg vessels after temperature equilibration. Curve 1, 1 micromole of  $\text{MnCl}_2$ . Curve 2, 1 micromole of  $\text{MnCl}_2$  and liver enzyme (specific activity 450) with 230  $\gamma$  of protein. Curve 3, 1 micromole of  $\text{MnCl}_2$  and liver enzyme (specific activity 450) with 460  $\gamma$  of protein. Curve 4, liver enzyme (specific activity 150) with 2.34 mg. of protein; no  $\text{MnCl}_2$ . Curve 5, same as Curve 4 but with 1 micromole of  $\text{MnCl}_2$ .

occurs. Under these conditions *l*-malic acid, which is formed in small amounts from pyruvic acid and  $\text{CO}_2$ , can be determined and identified by enzymatic methods.

Malic acid was determined with the pigeon liver enzyme, either by spectrophotometric measurement of the amount of TPN reduced or by determining the amount of  $\text{CO}_2$  evolved by the dismutation between malate and pyruvate (2). When pyruvate is present in large amounts, as is the case in the experiments to be described below, the direct spectrophotometric method gives values too low, mainly because of interference by the lactic dehydrogenase, which contaminates the pigeon liver enzyme. In this case the manometric method, which requires the presence of pyruvate, is more satisfactory. Pigeon liver enzyme of specific activity above 150 is of sufficient purity for the specific determination of *l*-malic acid under our experimental conditions.

Two experiments on hexose phosphate dismutation were carried out. The composition of the reaction mixtures is shown in Table VII.

TABLE VII

*Composition of Reaction Mixtures in Experiments on Dismutation between Glucose-6-phosphate and Pyruvate Plus  $\text{CO}_2$*

	Experiment 1	Experiment 2
Glucose-6-phosphate (sodium salt), micromoles.....	275	1100
Glucose-6-phosphate dehydrogenase ( <i>Zwischenferment</i> ), mg.....	12.5	50.0
TPN, micromoles.....	0.7	2.8
Sodium pyruvate, micromoles.....	275	1100
$\text{MnCl}_2$ , micromoles.....	5	20
Pigeon liver enzyme (specific activity 168), mg. protein....	0.88	3.5
Sodium bicarbonate, micromoles.....	400	800
Gas.....	80% $\text{N}_2$ -20% $\text{CO}_2$	
Final volume made up with water to.....	10.0 cc.	26.0 cc.

Pigeon liver enzyme was added last. The pH of the above reaction mixtures, after equilibration with  $\text{N}_2$ - $\text{CO}_2$ , was about 7.0. The samples were incubated at room temperature (22°) for 2 hours with occasional shaking. Samples for analysis (2.0 cc. each) were taken as follows: Experiment 1, at zero time just before addition of the liver enzyme, and after 1 and 2 hours of incubation; Experiment 2, after 2 hours of incubation. The remaining 24 cc. (Experiment 2) were used for isolation of malic acid as outlined below. Immediately after withdrawal, the samples taken for analysis were placed in a boiling water bath for 3 minutes, cooled, and centrifuged. The clear supernatants were used for determination of malic acid.

An approximate spectrophotometric determination of *l*-malate in the samples from Experiment 1 was carried out on 0.1 cc. aliquots of the supernatants, pigeon liver enzyme of specific activity 168, with 88  $\gamma$  of protein,

and 0.135 micromole of TPN<sub>ox.</sub> being used in each case. From the amount of TPN<sub>red.</sub> present when the reaction came to a standstill, it was calculated that the following amounts of *l*-malate were present in 10 cc. of reaction mixture: at time zero, 0.31 micromole; after 1 hour, 3.05 micromoles; after 2 hours, 5.0 micromoles. The results of the manometric estimations are shown in Table VIII. By this method, 8 micromoles of *l*-malate were found

TABLE VIII  
*Manometric Determination of l-Malate Formed by Reaction 5*

Determination based on the measurement of the CO<sub>2</sub> evolution caused by the reaction, *l*-malate + pyruvate → pyruvate + CO<sub>2</sub> + lactate, catalyzed by purified pigeon liver enzyme and lactic dehydrogenase in the presence of TPN and Mn<sup>++</sup>. 0.08 M acetate buffer, pH 5.2, 5 micromoles of MnCl<sub>2</sub>, 55 micromoles of pyruvate, 0.135 micromole of TPN, pigeon liver enzyme (free of fumarase, specific activity 168) with 440 γ of protein, and an excess of crystalline lactic dehydrogenase. Other additions as indicated. Final volume 2.5 cc. Gas, air; temperature, 38°. The reaction was started by tipping in pigeon liver enzyme from the side bulbs of Warburg vessels after temperature equilibration.

Time	CO <sub>2</sub> evolution, c.mm.						
	Blank	2.23 micro- moles <i>l</i> -malate	3.72 micro- moles <i>l</i> -malate	11.2 micro- moles <i>l</i> -malate	Experiment 1. 1.5 cc. supernatant before in- cubation	Experiment 1. 1.5 cc. supernatant after 2 hrs. incubation	Experiment 2. 1.5 cc. supernatant after 2 hrs. incubation
<i>min.</i>							
10				41	0	27	37
15	3	48	51				41
20				53	0	34	54
∞ *	6	55	86	242	0	34	61
CO <sub>2</sub> (corrected for blank), c.mm.		49	80	236		28	55
<i>l</i> -Malate found, micromoles		1.97	3.57	10.5	0	1.25	2.46
Total <i>l</i> -malate formed, micromoles						8.35	42.6

\* 30 to 150 minutes, depending on the amount of malate present.

in 10 cc. of reaction mixture in Experiment 1, and 42 micromoles of *l*-malate in 26 cc. of reaction mixture in Experiment 2, both after 2 hours of incubation. This corresponds to about 3 and 4 per cent respectively of the pyruvate added. It will be observed that the amounts of *l*-malate formed in each case are well in excess of the amount of TPN present, as required by Reaction 5.

For identification of the malic acid, the remaining 24 cc. of reaction mix-

ture from Experiment 2 were worked up as follows: The solution was cooled in ice, 5.0 cc. of ice-cold 10 per cent sodium bisulfite were added (to bind the pyruvate), and, after 5 minutes, the mixture was deproteinized with 8.0 cc. of ice-cold 15 per cent metaphosphoric acid. The protein precipitate was removed by centrifugation and the supernatant was extracted with ether continuously for 72 hours (11). The residue obtained after evaporation of the ether was dissolved in a little water, the solution was brought to pH 5.0 with ammonium hydroxide, precipitated with neutral lead acetate, and the lead precipitate was extracted with dilute nitric acid. The lead salt, obtained by bringing the pH of the nitric acid solution to 6.5 with dilute ammonium hydroxide, was redissolved in dilute nitric acid and reprecipitated at pH 6.5 as before. This was repeated once more. Finally the lead salt was suspended in a little water and decomposed with hydrogen sulfide. The solution was brought to pH 7.4 with dilute sodium hydroxide and made up with water to 3.5 cc. (Solution 1).

As a control, a 6.0 mg. sample (45 micromoles) of authentic *l*-malic acid was dissolved in water, precipitated with lead acetate at pH 5.0, and the precipitate was extracted with acid followed by fractionation of the lead salts as above. The final solution of free acid was brought to pH 7.4 and made up with water to 3.5 cc. (Solution 2).

Fig. 4 illustrates the results of the enzymatic experiments carried out with the above solutions. Curves 1, 2, and 3 show the presence of *l*-malic acid. Curve 4 is a control of the enzymatic determination of pyruvate with lactic dehydrogenase. Curve 5 shows that Solution 1 (0.04 cc. added at Arrow b) contained no pyruvate. Curve 6 shows that pyruvate was formed on incubation of an aliquot of Solution 1 with pigeon liver enzyme,  $Mn^{++}$ , and  $TPN_{ox}$ . This was done as follows: After completion of the reaction shown in Curve 3 (indicating the presence of 0.105 micromole of *l*-malic acid in 0.1 cc. of Solution 1) the reaction mixture (3.0 cc.) was acidified with 0.1 cc. of 2.0 N HCl and heated at 100° for 3 minutes;  $TPN_{red}$  is thus destroyed. After cooling, the mixture was brought back to pH 7.4 with 0.1 cc. of 2.0 N NaOH and centrifuged. 2.0 cc. of the clear supernatant, which should contain  $0.105 \times 2/3.2$ , or 0.066 micromole, of pyruvic acid, was mixed with an excess of  $DPN_{red}$  and crystalline lactic dehydrogenase. Curve 6 (Fig. 4) shows that 0.069 micromole of pyruvic acid was found.

According to the data of Fig. 4, 3.5 cc. of Solution 1, equivalent to 24 cc. of the reaction mixture of Experiment 2, contained 3.7 micromoles of *l*-malic acid or 4.0 micromoles for 26 cc. of reaction mixture. Since direct manometric determination gave 42.6 micromoles (Table VIII), the recovery (about 9.5 per cent) was very low. It should be observed, however, that the fractionation procedure, the main object of which was the complete removal of pyruvate, led to low recovery of the acid in the control with an

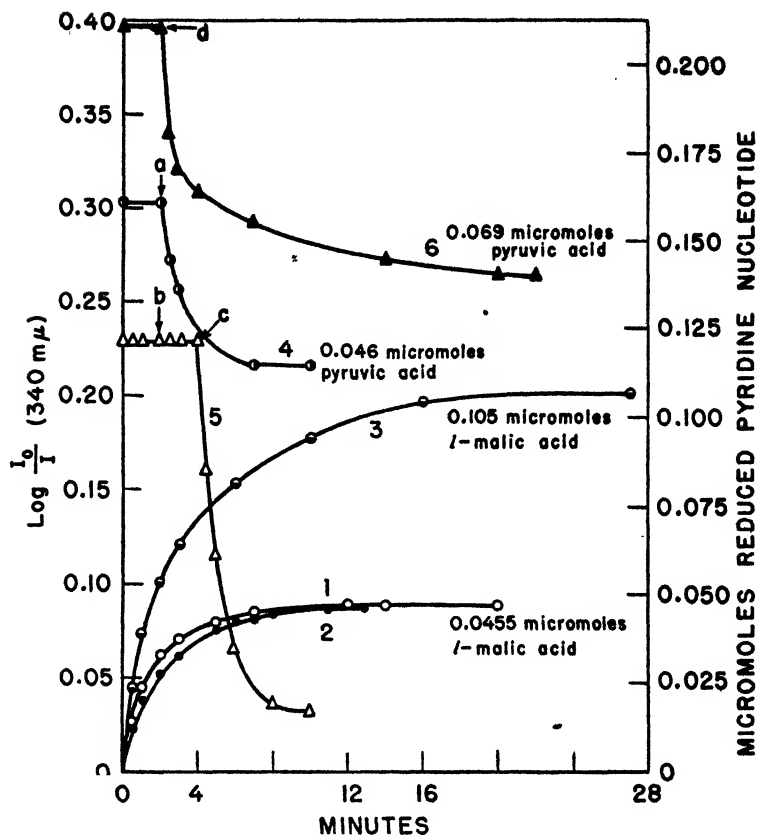


FIG. 4. Enzymatic identification of *l*-malic acid formed by dismutation between glucose-6-phosphate and pyruvate + CO<sub>2</sub>. 0.025 M glycylglycine buffer, pH 7.4, with other additions as indicated. Final volume, 3.0 cc.; temperature, 22°. Quartz cells; *d* = 1.0 cm. The blank cells contained no pyridine nucleotide. Malic acid estimations (Curves 1, 2, and 3), 1.0 micromole of MnCl<sub>2</sub>, either 0.135 (Curves 1 and 2) or 0.203 (Curve 3) micromole of TPN<sub>ox</sub>, and at time zero, liver enzyme (specific activity 168) with 132 γ of protein. Curve 1, 0.02 cc. of Solution 2 (see the text); Curve 2, 0.04 cc. of Solution 1; Curve 3, 0.1 cc. of Solution 1. Pyruvic acid estimations (Curves 4, 5, and 6), varying amounts of DPN<sub>red</sub>, and, at time zero, crystalline lactic dehydrogenase. Curve 4, 0.045 micromole of pyruvate added at Arrow *a*. Curve 5, 0.04 cc. of Solution 1 added at Arrow *b*; excess of pyruvate added at Arrow *c*. (The failure of the optical density to drop to zero is due to the presence of traces of hydrosulfite in the preparation of DPN<sub>red</sub>.) Curve 6, 2 cc. of filtrate from the experiment reproduced in Curve 3, present from the beginning; crystalline lactic dehydrogenase added at Arrow *d*. The amounts of *l*-malic acid or pyruvic acid found are given for each curve.

authentic sample of *l*-malic acid. Fig. 4 shows that 3.5 cc. of Solution 2 contained 8 micromoles of malic acid. Since 45 micromoles were fraction-

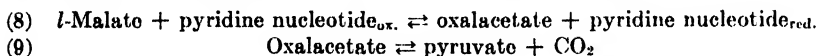


ated, the recovery was only 18 per cent. The control involved only fractionation of the lead salts without deproteinization and ether extraction.

#### DISCUSSION

It would appear that the reversible oxidative decarboxylation of malic acid is catalyzed either by a single enzyme or by enzymes which are so closely associated as to form a functional unit. There is some support for this in the data presented in the last column of Table V.

Reaction 1 is probably the over-all result of the individual Reactions 8 and 9. The fact that apparently the same enzyme is able to catalyze Reactions 1 and 9 would seem to support such a view.



However, we have no evidence that Reaction 8 is catalyzed by the liver enzyme, and attempts to obtain, in its presence, a reduction of oxalacetate by  $\text{TPN}_{red}$ , have so far been unsuccessful. One might, of course, think that, while the enzyme is able to decarboxylate synthetic oxalacetate, the real intermediate might be a form of oxalacetate which differs in some respect from the one obtained by synthesis. The fact that the enzyme is fully active in the absence of inorganic phosphate and ATP would seem to exclude phosphorylated intermediates, although the third phosphate in TPN might be involved in some as yet unknown manner. The specific stimulation by TPN of the decarboxylation of oxalacetate by the pigeon liver enzyme, which is not shared by the oxalacetic carboxylase of *Micrococcus lysodeikticus*, might be significant in this connection. Such an effect of TPN adds some support to the idea that both the over-all reaction and the decarboxylation of oxalacetate are catalyzed by one and the same enzyme or functional enzyme unit.

It is easy to visualize the failure of a mixture of malic dehydrogenase and oxalacetic carboxylase, each catalyzing Reactions 8 and 9 respectively, to catalyze the over-all reaction. Since the equilibrium of Reaction 8 is known to be far to the left, while that of Reaction 9 is far to the right, each would give rise from either side to minimum amounts of oxalacetate which would reach the surface of the other enzyme at too low a concentration to react at a significant rate. However, with a single enzyme involved, one could conceive that the intermediary oxalacetate might reach a sufficiently high local concentration to be metabolized in either direction right on the surface of the enzyme where it was produced.

While it is clear that the pigeon liver enzyme is responsible for the large fixation of  $\text{CO}_2$  in fumarate and malate observed by Evans *et al.* (5) and by Wood *et al.* (12), its relation to the fixation of  $\text{CO}_2$  in oxalacetate, occurring only in the presence of ATP (13, 6), is a matter for conjecture. TPN can-

not be substituted for ATP for such a fixation<sup>6</sup> (6). Thus, there might be a mechanism for fixation of CO<sub>2</sub> by pyruvate other than that involving the "malic" enzyme. Since the experiments on fixation of CO<sub>2</sub> in oxalacetate have so far been performed only with crude, or slightly purified, pigeon liver preparations, it would be desirable to carry out such experiments with purified oxalacetic carboxylase from *Micrococcus lysodeikticus*, which does not catalyze Reaction 1, and with highly purified preparations of the pigeon liver enzyme.

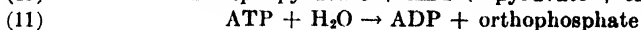
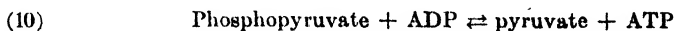
There seems to be some relationship of biotin to the liver enzyme (14); the nature of this relationship, however, is still obscure.

It may be significant that two systems of CO<sub>2</sub> fixation involving  $\beta$  carboxylation of an  $\alpha$ -keto acid, namely the isocitric and malic systems, both depend on TPN-specific enzymes for activity.

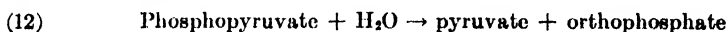
### Methods

\* Crystalline lactic dehydrogenase and malic dehydrogenase were prepared according to Straub (8, 10). Fumarase was purified from ox heart by the method of Laki and Laki (15) but it was not crystallized. The glucose-6-phosphate dehydrogenase (*Zwischenferment*) was kindly supplied by Dr. Erwin Haas.

The preparations of DPN and TPN were the same used in previous work (2, 3). The TPN preparation (purity, 54.6 per cent), which was free from inorganic phosphate, contained little or no adenosine polyphosphate (ADP, ATP) as shown by a sensitive spectrophotometric test. This test is based on the following reactions:



The enzyme catalyzing Reaction 10 has been isolated by Kubowitz and Ott (16); Reaction 11 is catalyzed by adenosinetriphosphatase. The net result is Reaction 12.



Reaction 12 requires the presence of catalytic amounts of ADP or ATP, but is not affected by DPN or TPN. The rate of this reaction is, within certain limits, a function of the concentration of adenosine polyphosphate. The rate of formation of pyruvate is determined, in the presence of DPN<sub>red.</sub> and lactic dehydrogenase, by spectrophotometric measurement of the rate of oxidation of DPN<sub>red.</sub> (Reaction 6). The results of these tests are illustrated in Fig. 5. An ammonium sulfate fraction from rabbit muscle extract, kindly supplied by Dr. E. Racker, contained the necessary enzymes, including lactic dehydrogenase.

\* Utter, M. F., and Wood, H. G., personal communication.

The preparation of  $\text{TPN}_{\text{red}}$  has already been described (2). Phosphopyruvate was kindly supplied by Dr. Gerhardt Schmidt. *l*-Malic and fumaric acids were obtained commercially. *d*-Malic acid was prepared

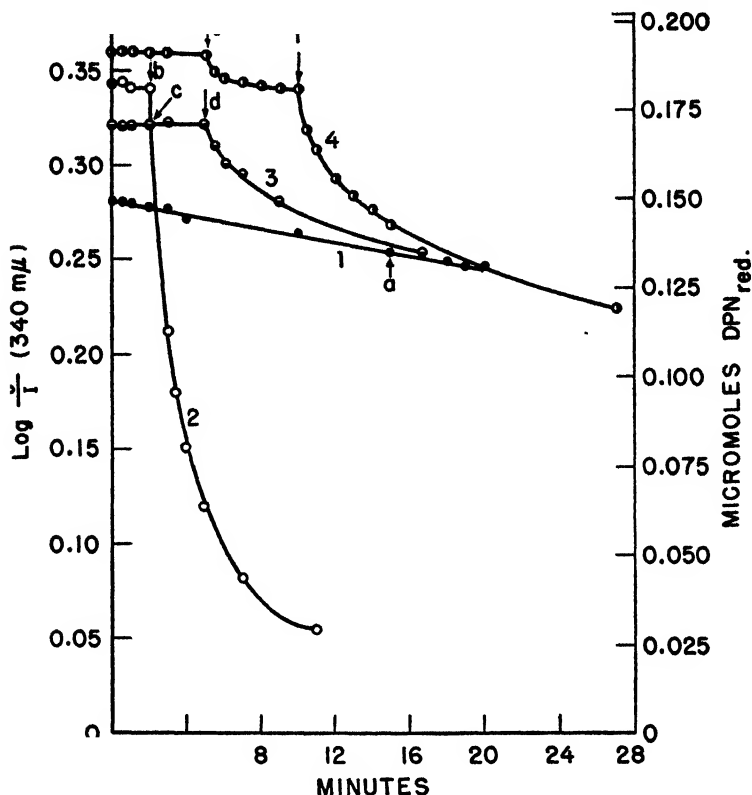


FIG. 5. Enzymatic test for ATP. 0.025 M glycylglycine buffer, pH 7.4, and varying amounts of  $\text{DPN}_{\text{red}}$ , with other additions as indicated. Final volume 3.0 cc.; temperature, 22°. Quartz cells;  $d = 1.0$  cm. The blank cells contained no DPN. Curve 1, crystalline lactic dehydrogenase and, at time zero, 2.7 micromoles of phosphopyruvate; at Arrow a, addition of 0.135 micromole of ATP. Curve 2,  $(\text{NH}_4)_2\text{SO}_4$  fraction from rabbit muscle extract (see the text) and, at time zero 1.35 micromoles of phosphopyruvate; at Arrow b, addition of 0.135 micromole of ATP. Curve 3, muscle fraction and, at time zero, 1.35 micromoles of phosphopyruvate; at Arrow c, addition of 0.0675 micromole of  $\text{TPN}_{\text{ox}}$ ; at Arrow d, addition of 0.0135 micromole of ATP. Curve 4, muscle fraction and, at time zero, 1.35 micromole of phosphopyruvate; at Arrow e, 0.0675 micromole of  $\text{TPN}_{\text{ox}}$ ; at Arrow f, 0.027 micromole of ATP.

from a commercial sample of *dl*-malic acid by resolution with cinchonine<sup>6</sup> according to Dakin (17).

<sup>6</sup> We are greatly indebted to Dr. B. B. Brodie for a generous supply of the alkaloid.

Highly specific methods for the determination of pyruvate and *l*-malate, with use of crystalline lactic dehydrogenase and the pigeon liver enzyme respectively, have been outlined in a previous section. The spectrophotometric method for malate gives satisfactory results with enzyme of specific activities between 150 and 200, provided that pyruvate is not present in too large amounts relative to malate. A 5-fold excess of pyruvate does not interfere. If higher relative amounts of pyruvate are present, the manometric method is preferable. However, the sensitivity of the former method is much higher, since about 5  $\gamma$  of *l*-malate can be accurately determined. By using purified fumarase in combination with the pigeon liver enzyme, *l*-malate and fumarate can be determined separately in a mixture containing both compounds.

#### SUMMARY

The partial purification and properties of a TPN-specific enzyme from pigeon liver, catalyzing the reversible oxidative decarboxylation of *l*-malate to pyruvate and CO<sub>2</sub>, and the decarboxylation of oxalacetate to pyruvate and CO<sub>2</sub> are described. The activity of this enzyme is markedly enhanced by manganous ions. While the evidence so far obtained suggests that a single enzyme, or functional enzyme unit, is involved, final decision on this point must await further purification.

The new enzyme probably plays a major rôle in the fixation of CO<sub>2</sub> by pyruvate in the liver. In analogy with the tricarboxylic acid system, the equilibrium of the over-all reaction, markedly in favor of decarboxylation, can be shifted in the opposite direction through a TPN-linked dismutation with the glucose-6-phosphate dehydrogenase system. The small amounts of *l*-malic acid formed from pyruvic acid and CO<sub>2</sub> under these conditions have been determined and identified by enzymatic methods.

Our thanks are due to Miss Marian L. Blanchard and Dr. J. B. Veiga Salles for help with some of the experiments. We are also indebted to Mr. Morton C. Schneider for technical assistance.

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## THE ACONITE ALKALOIDS

### XXI. FURTHER OXIDATION STUDIES WITH ATISINE AND ISOATISINE

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The mild oxidation of isoatisine,  $C_{22}H_{33}O_2N$ , with permanganate to a neutral derivative,  $C_{22}H_{33}O_3N$ , has been previously described (1). Further studies have shown that isoatisine and atisine can be oxidized to a mixture of acidic substances by more drastic procedures and the separation of several of these acids is reported here. The oxidation presumably passes through the neutral lactam stage such as oxoisoatisine, since the acids are not amphoteric and have given no indication of the retention of a basic N group.

Isoatisine has given a *tricarboxylic acid*,  $C_{21}H_{29}O_7N$ , in good yield. The additional oxygen is assumed to be present in the lactam grouping. The acid melts with evolution of approximately 1 M equivalent of  $CO_2$  (within 15 minutes). Since the acid was stable to heating with rather concentrated acid or alkali, decarboxylation of a malonic or acetoacetic acid type appears excluded. Both the acid and its thorium salt were pyrolyzed under a variety of conditions in attempts to obtain a cyclic ketone, the formation of which might have been the source of the  $CO_2$ . However, only small amounts of a resinous mixture resulted, from which nothing crystalline could be obtained. The study of such material was therefore inconclusive, although its neutralization equivalent indicated the retention of approximately two carboxyl groups.

The *trimethyl ester* obtained with diazomethane from the acid,  $C_{21}H_{29}O_7N$ , although non-crystalline, was purified by sublimation. It could be saponified quickly to a crystalline *monomethyl ester*. The latter proved to be relatively resistant to further saponification. The carboxyl group involved appears most probably to be of tertiary character. The monomethyl ester when heated above its melting point only very gradually yielded an appreciable amount of  $CO_2$ , and after an hour this amounted to about 0.5 M equivalent. This suggests that the tertiary carboxyl group may be the source of the  $CO_2$  in the case of the acid itself.

The tricarboxylic acid when refluxed with aniline yielded a derivative,  $C_{27}H_{35}O_4N_2$ , titration of which showed it to be a monocarboxylic acid. The compound must therefore be a cyclic *N-phenylimide* which indicates the proximity of the two carboxyl groups involved. On treatment of the

phenylimide with diazomethane, the imide ring was opened with the formation of a *dimethyl ester anilide*.

In an attempt to hydrogenate the tricarboxylic acid in methanol which contained HCl, hydrogenation did not occur but the substance recovered proved to be a *dimethyl ester*. In the latter, because of its ease of saponification to the original tricarboxylic acid, the tertiary carboxyl group could not have been involved. The dimethyl ester, however, now appeared sufficiently stable on heating to permit of its distillation unchanged.

The tricarboxylic acid was dehydrogenated with selenium to a mixture of phenanthrene hydrocarbons from which 1,6-dimethylphenanthrene was separated. If one excludes the unlikely production of a phenanthrene hydrocarbon by ring closure of a naphthalene fragment or the rather improbable conversion of a carboxyl to a methyl group during the dehydrogenation, the production of 1,6-dimethylphenanthrene is of special significance, since it has already been shown that 1-methyl-6-ethylphenanthrene is a dehydrogenation product of atisine (2). This indicates that in the production of the acid,  $C_{21}H_{20}O_7N$ , from isoatisine, the oxidation must have involved that portion of the molecule which gave rise to the ethyl group in methylphenanthrene.

The probable absence of a double bond in the tricarboxylic acid was indicated by the failure of attempts to hydrogenate it in methanol, in acetic acid, or in methanolic HCl. On such a basis, the formulation requires its tetracyclic character. Other evidence is against its unsaturated tricyclic character. If the latter were the case, the non-basic lactam N atom would be restricted to a side chain. But all attempts to hydrolyze the substance proved futile. It was recovered unchanged in excellent yield after treatment with 8 N HCl at 150° for 6 hours, after solution in concentrated  $H_2SO_4$  at room temperature for several days, after refluxing in 10 per cent NaOH for 6 hours, after fusion with molten alkali at 300°, and after treatment with the methyl Grignard reagent under forcing conditions. A simple amide linkage would be expected to be opened by such procedures. The observed stability of this linkage may be due to its inclusion in the fourth ring of the tricarboxylic acid, with a steric arrangement which is very resistant to cleavage.

An effort was made to determine whether the nitrogen was of secondary or of tertiary character. The substance was treated with bromine in methanolic NaOH under the conditions of the Hofmann degradation but was recovered unchanged. Similarly unsuccessful were attempts to brominate it in neutral or in acid solution. Because of the negative outcome of such experiments, the degree of substitution of the lactam nitrogen has not been determined. It appears certain, however, that the latter does not bear an ethyl group, since the 21 carbon atoms of the substance have been accounted

for in the following manner: 16 are present in the 1,6-dimethylphenanthrene fragment, 3 in the carboxyl groups, 1 in the lactam group, and 1 in the carbon atom lost during the oxidation of isoatisine to the  $C_{21}H_{29}O_7N$  acid. Although the original alkaloid gave about 50 to 60 per cent of the required amount in the N-alkyl determination, oxoisoatisine gave about 35 per cent and the tricarboxylic acid only about 19 per cent of the theoretical for N ethyl (determined as  $NCH_3$ ). Isoatisine under the conditions of the N-alkyl determination has previously been shown to yield ethyl iodide together with small amounts of methyl iodide (1). But the difficulties which we have encountered in the N-alkyl determinations with this class of substances have made it unsafe to base too much on the results obtained. There is a possibility that if atisine and isoatisine do not possess a simple N-ethyl group, the tertiary N could be contained in two rings in such a way that the source of the ethyl iodide is a two carbon bridge involving the N atom. The loss of two carbon fragments has already been indicated by the conversion of oxoisoatisine with methanolic HCl to an apparent  $C_{20}H_{29}O_3N$  derivative and by the production of a  $C_{20}H_{29}ON$  compound from atisine by catalytic dehydrogenation under high pressure (1). But the available data do not permit a final decision as to whether the so called N-ethyl group is involved in these transformations.

More recently, an apparent loss of a two carbon fragment accompanying partial decarboxylation of the tricarboxylic acid has also been noted. By treatment of the latter with either thionyl chloride or bromine and phosphorus tribromide followed by decomposition with water, a crystalline *dicarboxylic acid* was produced for which a possible formulation,  $C_{18}H_{25}O_5N$ , has been suggested by the analytical data.

The tricarboxylic acid does not appear to be a monosubstituted acetic acid,  $RCH_2COOH$ , because of the failure to brominate it under the conditions of the Hell-Volhard-Zelinsky method and because of its recovery unchanged when the Hunsdiecker reaction (3), *i.e.* the action of bromine on its silver salt, was attempted.

Atisine, when oxidized under the same conditions used for isoatisine, yielded a *dicarboxylic acid*,  $C_{21}H_{29}O_6N$ , in very small yield, together with oxalic acid. No oxalic acid was isolated from the oxidation of isoatisine described above. The acid which was separated from the oxidation mixture by the counter-current procedure of Craig (4) showed no basic properties and was therefore of lactam character. It proved to be stable above its melting point and could be sublimed unchanged. The saponification of its crystalline *dimethyl ester* to a more resistant *monomethyl ester* suggests the tertiary character of one of the carboxyl groups. The substance absorbed hydrogen in acetic acid and, although a crystalline product was obtained, its formulation remains uncertain and will be left to a later communication.



Like the tricarboxylic acid from isoatisine, this acid, from its formulation and ability to react with hydrogen, possibly contains four rings. It did not react with hydroxylamine and its absorption spectrum did not indicate the presence of a carbonyl group. By a more drastic oxidative procedure with permanganate, a  $C_{19}H_{27}O_6N$  tricarboxylic acid was also obtained from atisine. It was isolated in very small yield by the counter-current procedure.

From the facts at hand it is still premature to attempt to suggest definite structures for atisine and isoatisine. However, the data appear to permit of certain conclusions. There appears to be present a pentacyclic structure made up in part of a perhydrophenanthrene nucleus with a methyl group attached to position 1. Fused to this is a bicyclic structure containing the tertiary N atom. One point of attachment of this moiety to the perhydrophenanthrene nucleus appears to be at position 6; in isoatisine the hydroxyl groups and the two double bonds of the original alkaloids do not appear to be a part of the perhydrophenanthrene nucleus. One of the hydroxyl groups appears to be of a primary character and the source of a carboxyl group on oxidation. A ring which is outside of the perhydrophenanthrene nucleus and contains a double bond, and perhaps one of the hydroxyl groups, appears to be ruptured during the oxidation to the tricarboxylic acid.

#### EXPERIMENTAL

*Tricarboxylic Acid,  $C_{21}H_{29}O_7N$ , from Isoatisine*—5 gm. of isoatisine in 100 cc. of benzene were rapidly stirred with a solution of 1.25 gm. of NaOH in 100 cc. of water and cooled to 10°. At this temperature 15 gm. of powdered  $KMnO_4$  were added during 3 hours. An additional 5 gm. were added and the stirring continued overnight to complete the oxidation. The benzene was separated by centrifugation and yielded a negligible amount of resinous material. After removal of the  $MnO_2$ , the aqueous filtrate was strongly acidified with HCl. A doughy mass separated which was reduced to a powder on chilling and collected. After solution in a minimum of hot water and cooling, platelets separated. By concentration two successive fractions were obtained. The total yield was 2.6 gm. It decomposed at 258–261° with gas evolution.

$$[\alpha]_D^{25} = +9.3^\circ \text{ (c = 1.34 in methanol)}$$

$C_{21}H_{29}O_7N$ . Calculated. C 61.88, H 7.18, N 3.44, (N)CH<sub>3</sub> 3.68

Found. (a) " 62.04, " 7.04, " 3.45, " 0.70

(b) " 61.63, " 7.40, " 3.50

Neutralization equivalent (3COOH), calculated 135.7, found 139

176 mg. of the acid were heated in a sublimation apparatus at 300° for 15 minutes while a slow stream of CO<sub>2</sub>-free air was passed through the apparatus into a tower packed with glass beads containing 0.1 N Ba(OH)<sub>2</sub>.

Very soon the production of  $\text{CO}_2$  was evident. The temperature was lowered to  $120^\circ$  and the air stream was continued for 30 minutes. Titration with 0.1 N HCl showed that 0.85 equivalent of  $\text{CO}_2$  had been produced. An attempt was made to sublime the residue at 0.05 mm. from a bath at  $300^\circ$  but only 15 mg. of an amber resin were obtained.

100 mg. of the acid when melted and sublimed as rapidly as possible at  $320^\circ$  and 0.05 mm. yielded 35 mg. of a resin, C 62.20, H 7.03. A second experiment gave a resin which yielded C 62.93, H 7.50.

Various methods of fractionation yielded nothing crystalline from such material. When the resin was dissolved in a large volume of ether and concentrated, an amorphous powder separated. Analysis showed C 64.23, H 7.51; neutralization equivalent 176.

*Methyl Esters of Tricarboxylic Acid*—0.2 gm. of the  $\text{C}_{21}\text{H}_{29}\text{O}_7\text{N}$  acid dissolved in methanol was treated with an excess of diazomethane in ether. After removal of the solvent, the resinous residue of the trimethyl ester could not be crystallized and was sublimed at  $300^\circ$  and 0.05 mm. 130 mg. of a resin were obtained.

$[\alpha]_D^{20} = +9.6^\circ$ ( $c = 2.09$ in ethanol)			
$\text{C}_{24}\text{H}_{33}\text{O}_7\text{N}$ .	Calculated.	C 64.10, H 7.85, $\text{OCH}_3$ 20.71	
	Found.	" 64.43, " 7.89, " 19.61	

187.3 mg. of the trimethyl ester were heated at  $100^\circ$  in a mixture of 5 cc. of methanol and 13 cc. of 0.157 N NaOH. 0.5 cc. aliquots were withdrawn at intervals and back-titrated with 0.02 N acid. After 5, 30, and 100 minutes, the alkali consumed was 1.89, 2.06, and 2.13 equivalents. The solution was concentrated to a small volume and acidified. The resinous mass which separated crystallized on boiling. After recrystallization from water, 62 mg. of rod-shaped crystals of the monomethyl ester of the acid were obtained, which melted at  $210$ – $215^\circ$ .

$[\alpha]_D^{20} = +1.8^\circ$ ( $c = 1.67$ in ethanol)			
$\text{C}_{22}\text{H}_{31}\text{O}_7\text{N}$ .	Calculated.	C 62.67, H 7.42, N 3.33, $\text{OCH}_3$ 7.36, $(\text{N})\text{CH}_3$ 3.56	
	Found.	" 62.46, " 7.48, " 3.48, " 7.15 " 0.72	
Neutralization equivalent ( $2\text{COOH}$ ), calculated 210.6, found 222			

220 mg. of the monomethyl ester were heated in the apparatus as described above with the tricarboxylic acid itself. The substance melted at about  $200^\circ$ . Since no  $\text{BaCO}_3$  was observed, the temperature was raised to  $280^\circ$  in 15 minutes, at which point a slight precipitate of  $\text{BaCO}_3$  became evident. Heating was continued at this temperature for 60 minutes as the  $\text{BaCO}_3$  slowly increased. By titration it was shown that 0.52 M equivalent of  $\text{CO}_2$  had been produced. The residue, when subjected to a sublimation at  $280^\circ$  and 0.05 mm., yielded 70 mg. of a non-crystallizable resin. Found, C 64.53, H 8.12.

A rapid sublimation was performed by melting 200 mg. of the ester at 220° and then rapidly raising the temperature to 300° with reduction of the pressure to 0.02 mm. 170 mg. of a resin were obtained. Found, C 63.01, H 7.38. From dilute methanol 80 mg. were obtained crystalline; m.p. 200–210°. The melting point of a mixture with the original substance was 208–215°.

$C_{22}H_{21}O_7N$ . Calculated, C 62.67, H 7.42; found, C 62.48, H 7.52

2.1 gm. of the tricarboxylic acid were shaken for 3 days with 0.60 gm. of platinum oxide catalyst in 50 cc. of methanol which contained 2 cc. of HCl (1.19). The hydrogen absorption did not exceed that required by the catalyst. The substance recovered from the mixture proved to be a dimethyl ester and crystallized from methanol-water as stout rhombs which softened at 235° and melted at 240–245°.

$[\alpha]_D^{20} = +15.3^\circ$  ( $c = 2.02$  in ethanol)

$C_{22}H_{21}O_7N$ . Calculated. C 63.41, H 7.64,  $OCH_3$  14.26

Found. " 63.55, " 7.68, " 13.80

Neutralization equivalent, calculated, 435.27, found 410

200 mg. of the dimethyl ester were sublimed at 250° and 0.02 mm. and the sublimate was recrystallized from dilute methanol. 130 mg. of starting material were recovered which melted at 240–245°.

209 mg. of the dimethyl ester were heated at 100° with 14 cc. of 0.157 N NaOH. Samples were withdrawn after 5, 15, and 60 minutes and showed the consumption respectively of 2.95, 2.95, and 2.99 equivalents of alkali. After acidification of the mixture the tricarboxylic acid was recovered and melted with decomposition at 260–261°.

*Aniline Derivatives of Tricarboxylic Acid and Ester*—100 mg. of the  $C_{21}H_{20}O_7N$  acid were refluxed in 0.5 cc. of freshly distilled aniline for 30 minutes. Cold dilute HCl was added to the mixture and the solid material was collected. It was decomposed with dilute NaOH and the excess aniline was extracted with ether. The alkaline phase was acidified with HCl and the resulting gum was extracted with ethyl acetate. The extract after concentration yielded material which partially crystallized from ethanol-water. After repeated recrystallization from the same solvent, 30 mg. of stout needles were obtained which softened at 293° and melted at 299–302° with gas evolution.

\*  $C_{27}H_{22}O_6N_2$ . Calculated. C 69.79, H 6.95, N 6.03

Found. " 69.92, " 6.98, " 6.30

Neutralization equivalent, calculated 464.26, found 476

20 mg. of the aniline derivative were suspended in 2 cc. of methanol and an excess of diazomethane in ether was added. After an hour at room

temperature the solvents were removed and the residue was dissolved in ethyl acetate. The solution was shaken with 5 per cent NaOH, dried, and evaporated to dryness. The residue yielded from dilute methanol 15 mg. of fine needles which melted at 230–235°. After three recrystallizations, the melting point was 235–238°.

$C_{21}H_{29}O_7N_3$ . Calculated. C 68.19, H 7.50, N 5.49,  $OCH_3$  12.16  
Found. " 68.32, " 7.30, " 5.83, " 10.98

*Dehydrogenation of Tricarboxylic Acid*—A mixture of 2 gm. of the acid  $C_{21}H_{29}O_7N$  and 2.3 gm. of selenium was heated in a tube fitted with an air condenser. At 260° vigorous gas evolution accompanied by water condensation was noted. The temperature was raised to 320° for 7 hours. The ground reaction mass was repeatedly extracted with hot benzene. The benzene solution was shaken in turn with 10 per cent HCl and 5 per cent NaOH. The former was made alkaline with NaOH and extracted with ether. The latter yielded on concentration 50 mg. of a basic fraction. The NaOH extract after acidification with HCl yielded a negligible amount of acidic material. The benzene solution of the neutral fraction is described below.

The crude basic fraction was chromatographed on alumina (Merck) from benzene. By elution with benzene a zone which showed blue fluorescence under ultraviolet light was obtained. It consisted of 10 mg. of a colorless oil with a quinoline-like odor. The picrate was prepared and, after two recrystallizations from ethanol, 5 mg. of small yellow needles were obtained which melted at 311–316°. Further study of the substance has been deferred because of the amount available.

$C_{17}H_{22}N \cdot C_6H_5O_7N_3$ . Calculated, C 58.69, H 5.57; found, C 58.75, H 5.74

The benzene solution of the neutral fraction when concentrated to dryness yielded 0.51 gm. of a brown tar. This was chromatographed on alumina from benzene. On elution with benzene a band which fluoresced under ultraviolet light emerged and amounted to 0.11 gm. This was rechromatographed on alumina from petroleum ether. Elution with 10 per cent benzene in petroleum ether yielded 50 mg. of a colorless oil which crystallized on chilling. After two recrystallizations from ethanol, 20 mg. of plates were obtained which softened at 90° and melted completely at 105°. Since it was an obvious mixture, its further fractionation was studied.

A chromatograph on alumina with petroleum ether as solvent was prepared. The column was developed and eluted with 1:10 benzene-petroleum ether. As a broad fluorescing zone emerged, it was divided into two

fractions. The first fraction after recrystallization from ethanol melted at 85–95°.

$C_{16}H_{14}$ . Calculated, C 93.15, H 6.85; found, C 93.31, H 6.78

The second fraction yielded crystals which melted at 95–100°.

Found, C 93.32, H 6.63

The picrate of the first fraction was prepared and after two recrystallizations from ethanol melted at 140–141°. The hydrocarbon was regenerated

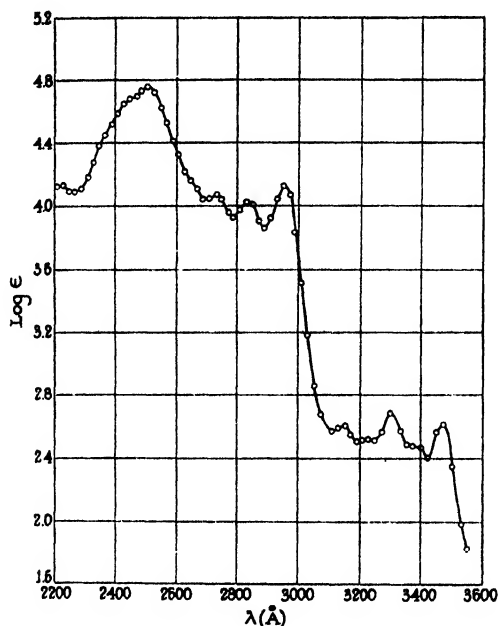


FIG. 1.  $C_{16}H_{14}$  hydrocarbon in ethanol

from the picrate by passing its ethanol solution through alumina. After recrystallization from ethanol, the hydrocarbon was obtained as plates which melted at 84–88°. The ultraviolet absorption spectrum is shown in Fig. 1. A sample of 1,6-dimethylphenanthrene, which was kindly sent to us by Professor R. D. Haworth, melted at 88–90°. A mixture of the two melted at 86–88°. The picrate of the purified dehydrogenation hydrocarbon crystallized from ethanol in yellow-orange needles which melted at 142–143°.

$C_{16}H_{14} \cdot C_6H_5O_7N_3$ . Calculated. C 60.66, H 3.93  
 Found. " 60.57, " 3.81

The melting point of a mixture with the picrate prepared from the authentic 1,6-dimethylphenanthrene was 142–143°. The trinitrobenzene addition product prepared from the hydrocarbons from both sources crystallized from ethanol in pale yellow needles and melted at 163–164°. No depression in melting point was noted with a mixture of the two substances.

*Action of Bromine-Phosphorus Tribromide and of Thionyl Chloride on Tricarboxylic Acid*—2 gm. of the tricarboxylic acid were mixed with 1 cc. of bromine, and 0.5 cc. of phosphorus tribromide was added with stirring. After standing overnight at room temperature, the mixture was heated on the steam bath for 1 hour and then boiled with 50 cc. of water. On cooling, a yellow resin formed. This was recrystallized in portions from water as described below for the thionyl chloride product. The substance separated as small aggregates of microscopic often triangular leaflets which melted at 220–223°.

$C_{18}H_{26}O_6N$ . Calculated. C 64.44, H 7.52, N 4.18

$C_{18}H_{27}O_6N$ . " " 64.05, " 8.07, " 4.15

Found. " 64.40, " 7.95, " 4.13

Neutralization equivalent, calculated 167.6, 168.6, found 185

150 mg. of the substance in methanol were treated with an excess of diazomethane. After removal of the excess reagent and solvent by concentration the residue was sublimed at 200° and 0.02 mm. 130 mg. of a resin were collected which could not be crystallized.

$C_{20}H_{26}O_6N$ . Calculated. C 66.07, H 8.05,  $OCH_3$  17.08

$C_{20}H_{31}O_6N$ . " " 65.71, " 8.55, " 17.00

Found. " 65.68, " 8.18 " 17.05

0.1 gm. of the tricarboxylic acid, when stirred with 1 cc. of purified thionyl chloride for 30 minutes, gradually dissolved. After warming to 60° for 10 minutes, excess reagent was removed *in vacuo*. The residue was boiled with 100 cc. of water and filtered from a yellow gum. The extract was concentrated to about 10 cc., when triangular leaflets separated which were larger than in the previous case. 30 mg. were collected. They lost birefringence at 200° and melted at 220° with decomposition.

Found. (a) C 64.11, H 7.97, N 3.91

(b) " 64.15, " 7.84

Neutralization equivalent, found 188

Methylation with diazomethane and distillation at 220° and 0.02 mm. yielded a resin which could not be crystallized and closely resembled in properties the ester described above. Found, C 65.78, H 8.39.

*Attempted Degradation of Tricarboxylic Acid with Bromine (3)*—200 mg. of the tricarboxylic acid were dissolved in an equivalent of dilute NaOH

and treated with a solution of 0.27 gm. of silver nitrate in 3 cc. of water with vigorous shaking. The gelatinous silver salt was collected, washed well with water, and then dried *in vacuo* over phosphorus pentoxide. The resulting 300 mg. were refluxed in 20 cc. of dry carbon tetrachloride and a solution of 0.2 M bromine in this solvent was added. 10 cc. were rapidly decolorized. An additional 10 cc. were added and the heating was continued for 30 minutes. The solid material was filtered off and the filtrate on evaporation yielded a negligible residue. The silver salt was decomposed with dilute HCl and in turn shaken with dilute NaOH. The filtrate from the solid material when acidified with HCl yielded a gum which was collected. On recrystallization from water 90 mg. of plates were obtained which melted with decomposition at 259–262° and proved in other ways to be unchanged tricarboxylic acid.

$C_{21}H_{29}O_7N$ . Calculated, C 61.88, H 7.18; found, C 61.47, H 7.05

*Dicarboxylic Acid*,  $C_{21}H_{29}O_6N$ , from Atisine—5 gm. of atisine hydrochloride were stirred rapidly in a mixture of 100 cc. of benzene and 100 cc. of  $H_2O$  which contained 1.25 gm. of NaOH. The temperature was kept at 10° and 8.6 gm. of  $KMnO_4$  were added in small portions during 3 hours. The mixture was then allowed to reach room temperature and an additional 9.4 gm. of  $KMnO_4$  were added in three portions. By morning the reagent had been completely used up. The aqueous and benzene phases were separated and the latter yielded only 50 mg. of a neutral resin which was discarded. The aqueous filtrate was acidified with HCl and 3.2 gm. of a resin were collected. The filtrate from this was concentrated to dryness and the residue was extracted repeatedly with hot ethyl acetate. The latter on slow concentration yielded large crystals. These were recrystallized from water. 0.23 gm. of oxalic acid dihydrate was obtained. The latter lost crystal water at 105–109° and decomposed with vigorous gas evolution at 195–196° and agreed in other properties with an authentic sample.

Neutralization equivalent, calculated 63.02, found 63.2

The 3.2 gm. of resin were subjected to a nine stage counter-current distribution in separatory funnels with, in each case, 50 cc. of ethyl acetate and 50 cc. of 2 M phosphate buffer (pH 5.9). After the distribution each funnel was made strongly acid with  $H_2SO_4$  and repeatedly extracted with ethyl acetate. The material from Funnels 5 and 6 partially crystallized after evaporation of the solvent. The crystalline material was collected with ethyl acetate and recrystallized from dilute ethanol. 0.2 gm. of rhombs was obtained which melted at 323–326° after preliminary softening at 310°.

$$[\alpha]_D^{25} = -23.5^\circ \text{ (c = 1.62 in ethanol)}$$

$C_{21}H_{27}O_4N$ .	Calculated.	C 64.41,	H 7.47,	(N)CH <sub>3</sub> 3.84
	Found. (a)	" 64.60,	" 7.33	" 1.12
	" (b)	" 64.37,	" 7.41	

Neutralization equivalent, calculated (2COOH) 195.62, found 193

After recrystallization from an ethanol-ethyl acetate-petroleum ether mixture, the melting point remained essentially unchanged.

Found, C 64.29, H 7.57

100 mg. of the acid were sublimed at 250° and 0.02 mm. 80 mg. of a partially crystalline resin were collected. Found C 64.37, H 7.60. When recrystallized from ethyl acetate it melted at 325–330°, and showed no depression when mixed with the original acid. Found C 64.30, H 7.41.

*Dimethyl Ester and Monomethyl Ester*—100 mg. of the dicarboxylic acid in acetone suspension were treated with excess diazomethane. After removal of solvent the residue was recrystallized from dilute ethanol. It formed needles; m.p. 193–195°.

$C_{23}H_{31}O_4N$ .	Calculated.	C 65.83,	H 7.93,	OCH <sub>3</sub> 14.80
	Found.	" 65.72,	" 7.89,	" 14.20

30.6 mg. of the dimethyl ester dissolved in 2 cc. of methanol were heated at 100° with 2 cc. of 0.157 N NaOH. Aliquots withdrawn after 1 hour and 1½ hours showed respectively the consumption of 0.99 and 1.04 equivalents of alkali. The solution was acidified and concentrated to small volume. Needles separated which were collected and recrystallized from dilute ethanol; m.p. 229–232°.

$C_{23}H_{31}O_4N$ .	Calculated.	C 65.14,	H 7.71,	OCH <sub>3</sub> 7.65,	(N)CH <sub>3</sub> 3.70
	Found.	" 65.07,	" 7.70	" 7.30,	" 0.73
		" 65.56,	" 7.72		

$C_{19}H_{27}O_6N$  Acid from Atisine—5 gm. of atisine hydrochloride were stirred with a solution of 1.5 gm. of NaOH in 50 cc. of H<sub>2</sub>O and 50 cc. of benzene. Without cooling 30 gm. of KMnO<sub>4</sub> were added in small portions during 2 hours. The aqueous phase was then separated. This was heated on the steam bath and an additional 10 gm. of KMnO<sub>4</sub> were added during 2 hours. The filtrate from MnO<sub>2</sub> was acidified with HCl. The filtrate from a small gummy precipitate was extracted three times with 25 cc. portions of ether which on evaporation yielded 1.07 gm. of a syrup. Continued ether extraction of the aqueous phase for 3 days yielded a mixture of crystals and some syrup. The crystals were collected with ether. After recrystallization from water 80 mg. of oxalic acid dihydrate were obtained which lost water at 105–109° and melted with decomposition at 195–196°.



The above 1.07 gm. of syrup were subjected as described above to a nine-stage Craig counter-current distribution between 50 cc. phases of ether and 2 M phosphate buffer (pH 5.3) in separating funnels. After the distribution each funnel was acidified (pH 3) with phosphoric acid and the aqueous phase was extracted repeatedly with ether. Each fraction was then examined. The material from Funnels 4, 5, and 6 partially crystallized on removal of the ether. That from Funnel 5 was collected with ether and, when recrystallized from ethanol-ether, yielded 15 mg. of very small prisms. The substance softened at 205°, then resolidified in a different crystalline form and again melted at 310–315°. From Funnels 4 and 6, 25 mg. of the same substance were obtained. The melting point of a mixture of this substance with the above dicarboxylic acid, (C<sub>21</sub>H<sub>29</sub>O<sub>6</sub>N, from atisine showed a definite depression (m.p. 285–293°).

$$[\alpha]_D^{25} = +39^\circ \text{ (} c = 1.08 \text{ in ethanol)}$$

C<sub>21</sub>H<sub>29</sub>O<sub>6</sub>N. Calculated. C 62.43, H 7.45, N 3.84, (N)CH, 4.11

Found. " 62.35, " 7.14, " 3.82, " 0.00

Neutralization equivalent, calculated (3COOH) 121.74, found 127

All analytical data have been obtained by Mr. D. Rigakos of this laboratory.

#### SUMMARY

A further study of the oxidation of isoatisine with permanganate has yielded a tricarboxylic acid, (C<sub>21</sub>H<sub>29</sub>O<sub>7</sub>N, which also contains the lactam grouping. The tertiary character of one of its carboxyl groups has been suggested by the behavior of the methyl esters with alkali. Dehydrogenation of this acid has yielded 1,6-dimethylphenanthrene which conforms with the production of 1-methyl-6-ethylphenanthrene from atisine itself.

Further study of the oxidation of atisine with permanganate has yielded a dicarboxylic acid, C<sub>21</sub>H<sub>29</sub>O<sub>6</sub>N, and a tricarboxylic acid, C<sub>19</sub>H<sub>27</sub>O<sub>6</sub>N.

From the data obtained certain tentative conclusions have been drawn in regard to a number of structural features of atisine.

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# THE GROWTH REQUIREMENTS OF CLOSTRIDIUM PERFRINGENS (WELCHII) BP6K\*

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The objectives of the investigation of the nutrition of *Clostridium perfringens* were twofold. In the first place, the development of a chemically defined medium was prerequisite for the understanding of the factors and conditions favorable to production of toxins or exoenzymes. Secondly, it became evident early in the investigation that the organism presented exceptional advantages as a means of assay for amino acids and vitamins. The fact that very dense growth is obtained in 16 hours at 45° in the presence of a reducing agent and the fact that thirteen amino acids and four vitamins are absolute requirements of the organism pointed to the probable merits of the bacterium for their assay.

The early experiments in this laboratory on the growth requirements of *Clostridium perfringens* demonstrated that the organism would grow in a medium consisting of vitamin-free casein hydrolysate, tryptophan, cystine, glucose, vitamins (biotin, calcium *d*-pantothenate, riboflavin), uracil, adenine, inorganic salts, and an extract of liver. Later, the organism was grown successfully on a medium which had the same composition as the one above except that chemically pure amino acids were substituted for the casein hydrolysate and pyridoxamine (or pyridoxal) for the extract of liver.

The chemically defined medium to be described for *Clostridium perfringens* BP6K consists of nineteen known amino acids, uracil, adenine, glucose, vitamins (biotin, calcium *d*-pantothenate, riboflavin, pyridoxamine), and salts. This medium supports rapid and luxuriant growth of the organism from a small inoculum. The growth obtained is equal to that produced on a medium prepared from enzymatic digests or hydrolysates of tissues or proteins supplemented with essential requirements. Under the conditions of the experiments there is no formation of  $\alpha$  toxin (lecithinase) or any of the other known toxins or exoenzymes (hyaluronidase,  $\theta$  toxin, gelatinase) in this medium. However, this organism does not lose its ability to form toxins when grown under conditions suitable for

\*The work described in this paper was assisted by the United States Army.

toxin production (1). In addition to strain BP6K, five other strains<sup>1</sup> of *Clostridium perfringens*, A224B, A225B, G6000, G6006, and F5022, have been grown successfully in this medium.

The organism requires nine of the ten amino acids said to be essential for the rat (2), in addition to glutamic acid, serine, tyrosine, cystine, and the vitamins, pyridoxamine, calcium *d*-pantothenate, biotin, and riboflavin.

The relation of the lysine, alanine, aspartic acid, and glycine requirements to the pyridoxine, pyridoxamine, or pyridoxal content of the medium will be discussed.

### Procedure

Stock cultures of the organism are maintained by serial transfers in a stock medium consisting of casein hydrolysate, with added tryptophan, cystine, adenine, uracil, vitamins, glucose, salts, phosphate buffer, and defatted beef heart. Inoculum for the chemically defined medium is grown in pancreatic digest of beef heart (1) or tryptic digest of casein plus salts and vitamins. On a few occasions the inoculum has been grown in Bacto-tryptose or Bacto-peptone with added salts and vitamins, but on these media the organisms become ropy (mucoïd) if stored (4–6°) for more than 1 day. These cultures are seed cultures.

*Stock Cultures*—The medium for the stock cultures of the organism *Clostridium perfringens* BP6K is prepared as follows:

Enough casein hydrolysate (Difco casamino acids) to contain 1.6 gm. of nitrogen (usually 16 gm. of solids), 5.76 gm. of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 0.48 gm. of  $\text{KH}_2\text{PO}_4$ , 0.02 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02 gm. of *L*-cystine, 0.02 gm. of *L*-tryptophan, and 5.0 gm. of sodium succinate is dissolved in 500 cc. of water. The solution is adjusted to pH 7.7 to 7.8 with sodium hydroxide.

5 cc. of a stock vitamin solution which contains 1 mg. each of calcium *d*-pantothenate and pyridoxine and 0.1 mg. of riboflavin are added and the solution is made to 1 liter. The medium is distributed in 25 × 200 mm. Pyrex culture tubes, 40 cc. to each tube.

To each tube is added 1 gm. of dry fat-free beef heart which is prepared as follows: Beef heart freed from gross fat is ground in a meat chopper. 1 kilo is thoroughly mixed with 3 liters of 95 per cent alcohol. The tissue is kneaded with the alcohol several times during the course of 4 hours. The alcohol is then removed as well as possible by filtering and pressing the tissue in a cloth bag. The tissue is kneaded in 3 liters of 1:1 alcohol-ether mixture. After 4 hours, the alcohol-ether is drained off and the tissue dried by spreading in a thin layer in a current of air. The dried tissue is reground in a coffee mill to particles 2 mm. or less in diameter.

<sup>1</sup> *Clostridium perfringens* BP6K was obtained from the National Institute of Health, Bethesda, Maryland. The British strains A224B, A225B, G6000, G6006, and F5022 were obtained from the Lister Institute, Elstree, Herts, England.

The tubes containing 1 gm. of beef heart per 40 cc. of medium are then autoclaved at 10 pounds for 30 minutes.

This complete stock culture medium is cooled to below 38° in a water bath, 1 cc. of sterile 25 per cent glucose is added to each tube, and inoculation made without delay. The tubes are incubated for 5 to 6 hours at 38° and stored at 4–6° for periods up to 1 month.

*Tryptic Digest of Casein*—Seed culture medium has been prepared in this laboratory from tryptic digest of casein with added glucose. 1 kilo of casein is suspended in 8 liters of distilled water warmed to 50°, and the pH is adjusted to approximately 8.0 with NaOH solution. 30 gm. of trypsin (1:100) are suspended in water to make a smooth flowing paste or slurry and are poured into the casein. The digest is thoroughly mixed with 200 cc. of toluene and placed in an incubator at 45–50° for 24 hours. At the completion of the digestion the digest is placed in the cold room (4–6°) overnight and filtered with the aid of Filter-Cel. The digest is adjusted to pH 5.5 and allowed to boil for 10 minutes, after which it is filtered by suction with the aid of Filter-Cel.

To prepare 1 liter of medium, an amount of tryptic digest which contains 40 gm. of solid is measured out. To this is added the amount of salts and vitamins required for a liter of stock culture, except that cystine, tryptophan, and sodium succinate are omitted. The volume of the solution is made to 1 liter. The pH is adjusted to 7.6 with NaOH.

The medium is distributed in 25 × 200 Pyrex culture tubes, 40 cc. to a tube, and autoclaved at 10 pounds for 30 minutes. The tubes are cooled to below 38° in a water bath, 1 cc. of 25 per cent sterile glucose solution is added to each, and they are then inoculated without delay with 1 cc. of stock culture. The tubes which are not to be inoculated immediately are stored at room temperature without the addition of glucose. Just before inoculation they must be heated in a boiling water bath for 20 minutes to expel dissolved air, then cooled quickly, and the glucose added. The inoculated tubes are incubated for 5 to 6 hours at 38° and stored at 4–6° for periods up to 1 week.

*Inoculum*—1 cc. of seed culture is centrifuged under sterile conditions and the supernatant discarded. The packed cells are mixed with 10 cc. of sterile saline and centrifuged again. The washed cells are then resuspended in 1 cc. of sterile saline and diluted at least as much as 1:5 × 10<sup>5</sup> and may be diluted as high as 1:3 × 10<sup>8</sup> (see Table V) with saline. 1 cc. of the diluted inoculum is added per tube of medium.

*Chemically Defined Medium*—The composition of 100 cc. of double strength basal medium consisting of amino acids, a purine and pyrimidine, salts, vitamins, and glucose is shown in Table I. The medium is prepared as follows: The amino acids (with the exception of cystine) plus ascorbic acid, shown in Table I, are weighed and kept in a dry flask until

ready for use. Cystine is weighed separately and dissolved with the aid of a few drops of  $N$  HCl.

50 mg. of uracil and 87 mg. of adenine sulfate are dissolved in 50 cc. of 0.2  $N$  HCl. 2.5 cc. of this solution are required.

10 gm. of  $MgSO_4 \cdot 7H_2O$ , 0.5 gm. of  $FeSO_4 \cdot 7H_2O$ , 0.5 gm. of NaCl, and 0.5 gm. of  $MnSO_4 \cdot 4H_2O$  are dissolved, 1 cc. of syrupy  $H_3PO_4$  is added, and the mixture made to 250 cc. with water. 1 cc. of this solution is required.

TABLE I  
*Double Strength Basal Medium*

Glucose	2.0 gm.	Riboflavin	100.0 $\gamma$
Ascorbic acid	50.0 mg.	Ca <i>d</i> -pantothenate	200.0 "
DL-Alanine	100.0 "	Pyridoxamine dihydrochloride	100.0 "
D-Arginine*	50.0 "	Biotin	1.0 "
DL-Aspartic acid	100.0 "	$MgSO_4 \cdot 7H_2O$	40.0 mg.
L-Cystine*	20.0 "	$FeSO_4 \cdot 7H_2O$	2.0 "
Glycine	100.0 "	$MnSO_4 \cdot 4H_2O$	2.0 "
L-Glutamic acid*	150.0 "	NaCl	2.0 "
L-Histidine*	50.0 "	Adjust to pH 7.2 with NaOH	
Hydroxy-L-proline	25.0 "	$K_2HPO_4$	1.66 gm.
DL-Isoleucine*	50.0 "	$KH_2PO_4$	0.32 "
L-Leucine*	75.0 "	Add distilled $H_2O$ to 100.0 cc.	
L-Lysine*	100.0 "		
DL-Methionine*	50.0 "		
L-Proline	25.0 "		
DL-Phenylalanine*	50.0 "		
DL-Serine*	150.0 "		
DL-Threonine*	50.0 "		
L-Tryptophan*	50.0 "		
L-Tyrosine*	50.0 "		
DL-Valine*	75.0 "		
Uracil	2.5 "		
Adenine sulfate	3.4 "		

\* Essential for growth.

10 mg. of riboflavin and 20 mg. of calcium *d*-pantothenate are dissolved and made to 100 cc. with water. 1 cc. of this solution is required.

20 mg. of pyridoxamine dihydrochloride are dissolved and made to 100 cc. with water and kept in a dark bottle at 4–6°. 0.5 cc. of the solution is required.

A vial of biotin containing 25  $\gamma$  is made to 25 cc. with distilled water. 1 cc. of the solution is required.

Phosphate buffer is prepared by dissolving 52 gm. of  $K_2HPO_4 \cdot 3H_2O$  and 10 gm. of  $KH_2PO_4$  in distilled water and making the solution to 250 cc. 8 cc. of the solution are required.

60 cc. of water are added to the dry amino acids and ascorbic acid. The mixture is heated in a boiling water bath with occasional shaking until solution is complete. The solution is cooled and cystine, uracil plus adenine, salts, and vitamin solutions are added and the pH adjusted to 7.1 to 7.2 with NaOH. The phosphate buffer solution is then added and the volume of the mixture made to 100 cc. The final pH should be 7.1 to 7.2.

This double strength basal medium is diluted with an equal volume of water for final use. The medium may be sterilized (without glucose) and kept for at least a week with satisfactory results.

*Procedure for Growth*—The procedure for obtaining maximum growth on the chemically defined medium is as follows:

5 cc. quantities of the double strength basal medium without glucose are pipetted into culture tubes (15 × 150 mm.) and the final volume is adjusted to 10 cc. The tubes are covered with loosely fitting glass caps, or stoppered with cotton plugs in the usual manner.

The tubes are sterilized for 15 minutes at 15 pounds pressure, cooled, and 0.4 cc. of sterile 25 per cent glucose added. Each tube is inoculated with 1 cc. of washed, diluted, bacterial cell suspension and then placed in a constant temperature water bath at 45° for 16 to 20 hours, depending on the density of the inoculum. Top growth may be obtained in 16 hours when  $5 \times 10^{-4}$  cc. of original culture are used for inoculum, but if a very small inoculum ( $3.2 \times 10^{-9}$  cc. of culture) is employed, it is necessary to extend the incubation period to 20 hours. At the end of the incubation period the contents of each tube are mixed and the very heavy growth ( $1.7 \times 10^9$  organisms per cc.) is measured with a turbidimeter designed for dense suspensions (3). An Evelyn photoelectric colorimeter has also been employed for measuring turbidity and direct cell counts have been made. Measurements made by these three means are the criteria used for the estimate of growth as defined in this paper.

#### RESULTS AND DISCUSSION

Table I shows the amount of amino acids required per 100 cc. of double strength medium for maximum growth of *Clostridium perfringens*. Thirteen amino acids are absolute requirements for this organism. Nine of these amino acids are indispensable for the rat (2): arginine, leucine, histidine, isoleucine, methionine, threonine, phenylalanine, tryptophan, and valine. The other amino acids essential for growth of the organism are glutamic acid, serine, cystine, and tyrosine. Those amino acids which appear to be non-essential for growth, but which are added to the medium because of their stimulating effect on growth, are glycine, alanine, lysine, aspartic acid, proline, and hydroxyproline.

As far as it is known at the present time, only the natural forms of the

amino acids are used by this anaerobe. The growth response of the organism to the L form of valine, serine, tryptophan, and methionine is twice that with the DL form.

Table II shows the effect of the omission of single amino acids from an otherwise complete medium on the growth of *Clostridium perfringens*. The omission of any one of the thirteen amino acids which are essential results in little or no growth after incubation for 16 hours at 45°.

In the beginning, the amount of each essential amino acid to be added to the medium was determined on the basis of the amount present in vitamin-free casein hydrolysate. Later, by adding graded amounts of each essential amino acid to an otherwise adequate mixture, the minimum amount which would produce top growth was determined. The amount

TABLE II  
*Growth of Clostridium perfringens Obtained When One Amino Acid Was Omitted from Otherwise Complete Medium*

Amino acid omitted	Turbidity*	Amino acid omitted	Turbidity*
L-Arginine	0.2	L-Glutamic acid	0
DL-Isoleucine	0.2	DL-Serine	1.0
L-Leucine	0	L-Tyrosine	0.8
DL-Methionine	0.2	L-Lysine	15.0
DL-Phenylalanine	0.1	DL-Alanine	15.0
DL-Threonine	0.2	L-Proline	17.0
DL-Valine	0.1	Hydroxy-L-proline	16.5
L-Tryptophan	0	DL-Aspartic acid	16.0
L-Histidine	0.4	Glycine	15.0
L-Cystine	0.2		

Incubated 16 hours at 45°.

\* Microamperes.

subsequently added to the basal medium was then arbitrarily set at 4 times this quantity.

The importance of the members of the vitamin B<sub>6</sub> group in the synthesis of certain amino acids by other microorganisms has been reported by Stokes *et al.* (4), Lyman *et al.* (5), and Speck and Pitt (6). Stokes *et al.* have shown that if the pyridoxine of the medium is replaced with either pyridoxamine or pyridoxal the requirement of *Lactobacillus delbrueckii*, *L. casei*, and *L. arabinosus* 17-5 for lysine, threonine, and alanine is eliminated.

Lyman *et al.* showed that pyridoxine could replace lysine, threonine, and alanine in the medium for *Lactobacillus arabinosus* 17-5, and furthermore that carbon dioxide and pyridoxine together would replace arginine, phenylalanine, and tyrosine.

Speck and Pitt have reported that pyridoxamine or pyridoxal in the medium eliminates the requirement of *Lactobacillus arabinosus* 17-5 for cystine.

Likewise, pyridoxamine or pyridoxal (or very large amounts of pyridoxine) apparently eliminates the requirement of *Clostridium perfringens* for lysine, alanine, aspartic acid, and glycine.

Fig. 1 shows the response of *Clostridium perfringens* to the different members of the vitamin B<sub>6</sub> group when lysine is omitted from the medium. Apparently lysine is not required by the organism when pyridoxamine

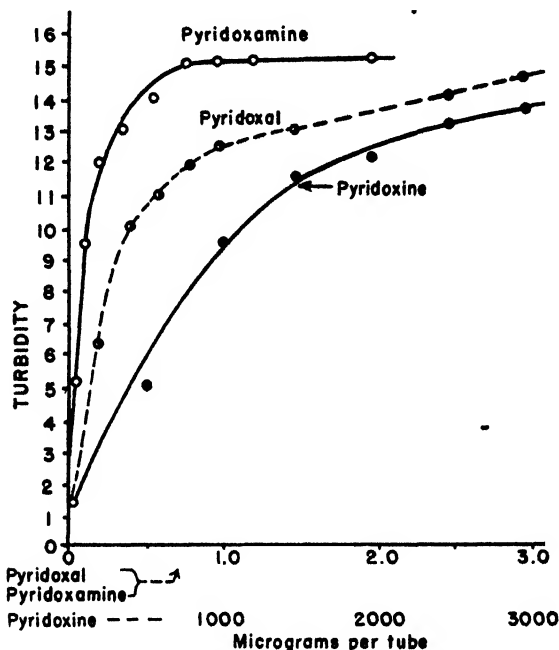


FIG. 1. Growth response of *Clostridium perfringens* to pyridoxine, pyridoxal, and pyridoxamine on a medium free of lysine.

(or pyridoxal) is present to the extent of 1 to 3  $\gamma$  per 10 cc. of medium. However, to obtain nearly the same growth response with pyridoxine, it must be added in amounts 2000 to 3000 times that of pyridoxamine. Similarly, Snell and Rannefeld (7) have shown that the activity of pyridoxamine and pyridoxal for a large group of lactic acid bacteria is from a few fold to several thousand fold that of pyridoxine.

Table III also demonstrates that aspartic acid, glycine, and alanine, respectively, are very likely synthesized when pyridoxamine or pyridoxal or very large amounts of pyridoxine are present in the medium.



TABLE III

*Effect of Pyridoxine, Pyridoxamine, and Pyridoxal on Growth of Clostridium perfringens in Medium Deficient in Alanine, Aspartic Acid, or Glycine*

	Pyridoxine		Pyridoxamine		Pyridoxal	
	$\gamma$ per tube	Turbidity*	$\gamma$ per tube	Turbidity*	$\gamma$ per tube	Turbidity*
No alanine	500	0.5	0.10	0.5	0.5	0.6
	2500	11.0	0.40	14.2	3.0	15.5
4 mg. alanine per tube	0	0.2	0	0.2	0	0.2
	50	9.5	0.05	12.6	0.1	4.5
	250	15.5	0.10	15.5	0.5	17.0
No aspartic acid	40	3.5	0.04	5.0	0.20	9.0
	200	11.0	0.20	15.0	0.60	13.5
5 mg. aspartic acid per tube	0	0.2	0	0.2	0	0.2
	40	6.5	0.04	9.0	0.20	13.5
	60	11.0	0.06	12.5	0.30	15.0
No glycine	20	4.0	0.05	12.0	0.15	8.0
	40	10.2	0.08	15.0	0.60	16.2
4 mg. glycine per tube	0	0.7	0	0.7	0	0.7
	20	10.5				
	40	13.5				

\* Microamperes.

TABLE IV

*Acceleration of Growth by Adenine, Uracil, and Guanine*

Adenine sulfate	Guanine HCl	Uracil	Turbidimetric reading, microamperes							
			Incubation time							
			2 hrs.	4 hrs.	6 hrs.	8 hrs.	10 hrs.	13 hrs.	16 hrs.	25 hrs.
$\gamma$ per tube	$\gamma$ per tube	$\gamma$ per tube								
0	0	0	0	0	0	+	0.1	6.5	10.0	17.0
100			0	+	3.5	10.0	11.0	13.5	16.0	18.5
200			0	+	6.0	11.0	12.0	14.5	17.5	18.7
	100		0	0	+	2.0	9.5	13.5	16.5	19.0
	200		0	0	0.5	6.0	10.0	13.0	16.0	18.5
		100	0	0	+	+	0.5	7.0	10.0	16.0
		200	0	0	+	+	0.6	8.0	10.5	16.5
200		20	+	1.5	9.5	12.5	13.5	16.0	17.5	19.5
200		100	+	2.0	9.5	12.5	14.0	16.5	17.5	19.5
	200	100	0	0	+	1.0	8.2	11.5	14.0	18.0
	200	200	0	0	0.5	8.5	11.5	14.0	16.5	18.5

+ indicates easily visible growth.

Table IV shows the effect of added adenine, guanine, and uracil on the induction period of the organism when incubated at 45°. When these

three compounds are omitted from the medium, there is an induction period of 10 hours before growth becomes evident. The addition of adenine enables visible growth to take place in 4 hours after inoculation. A still more marked acceleration of growth is obtained by adding uracil and adenine. Growth of the organism is readily visible in  $1\frac{1}{2}$  to 2 hours after inoculation with  $5 \times 10^{-4}$  cc. of the original seed culture. Apparently synthesis of purines takes place at a slow rate, for after 16 hours incubation only half maximum growth has occurred in a medium free of guanine and adenine. Guanine and uracil also reduce the induction period but not to the same extent as adenine and uracil.

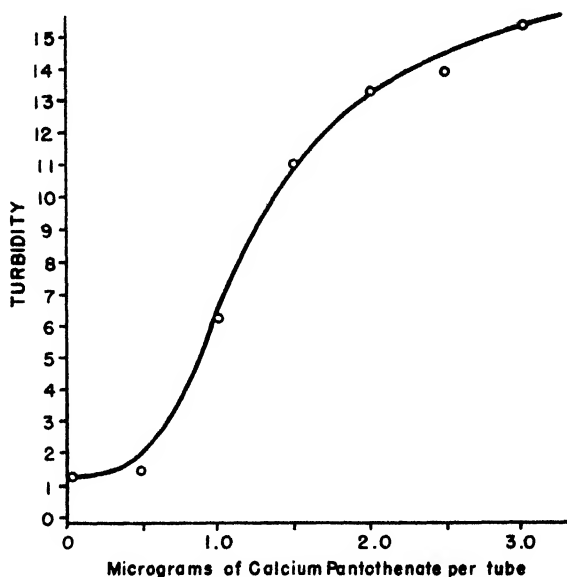
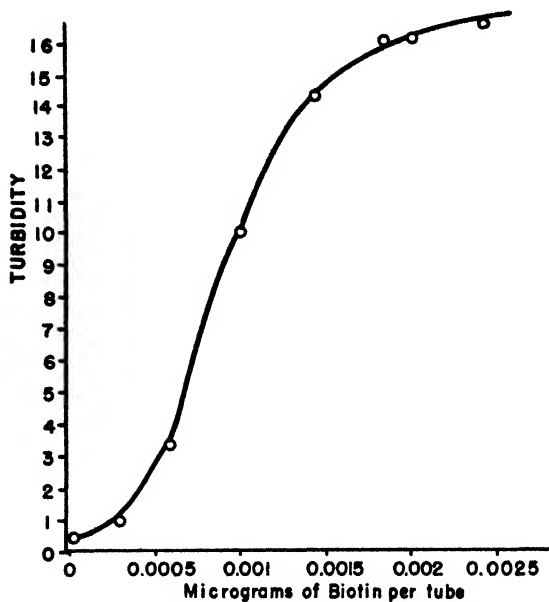
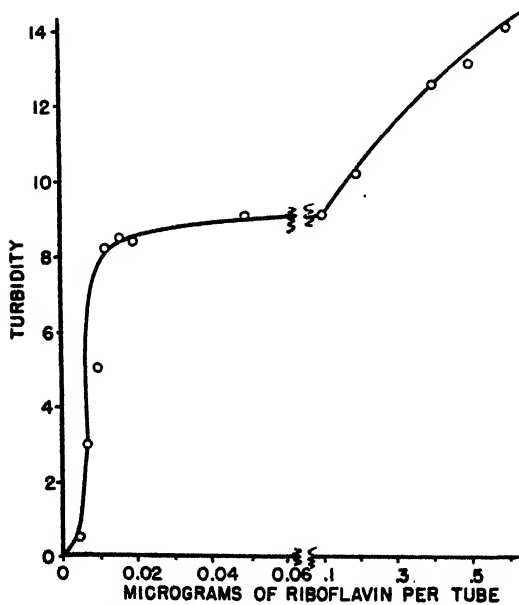


FIG. 2. Effect of calcium *D*-pantothenate on the growth of *Clostridium perfringens*

The only vitamins required for growth by *Clostridium perfringens* are calcium *D*-pantothenate, pyridoxamine (or pyridoxal), biotin, and riboflavin. Fig. 2 illustrates the response of the organism to increments of calcium *D*-pantothenate in an otherwise complete medium. Maximum growth is obtained in a medium containing 3  $\gamma$  of calcium *D*-pantothenate per tube.

The response of the organism to biotin is shown in Fig. 3. Maximum growth is obtained in a medium containing 0.003  $\gamma$  per tube.

Fig. 4 shows the growth with the addition of riboflavin to an otherwise adequate medium. Growth is visible with the addition of 0.005  $\gamma$  of riboflavin and increases to half maximum growth on the addition of 0.015  $\gamma$ .

GROWTH REQUIREMENTS OF *C. PERFRINGENS*FIG. 3. Effect of biotin on the growth of *Clostridium perfringens*Fig. 4. Effect of riboflavin on the growth of *Clostridium perfringens*

With the addition of larger amounts the increments of response become progressively smaller until 0.7 to 1.0  $\gamma$  is required to produce top growth.

The "B" salts, which consist of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , and  $\text{NaCl}$ , are added in the same amounts as are required by the lactobacilli. Mg and Fe are essential requirements.

The phosphate buffer, which is a mixture of  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$ , is added in large amounts so as to keep the pH high during growth. When pH 4.5 is reached, the organism ceases to grow.

The concentration of glucose in the medium is 1 per cent and may be increased to 2 per cent without affecting the growth of the organism.

If the medium is autoclaved before adding glucose, it is necessary to add a reducing substance such as ascorbic acid (either *d* or *l* isomer) or cysteine hydrochloride to produce an oxidation-reduction potential sufficiently low to initiate the growth of this anaerobe. Ascorbic acid is preferred as the reducing agent because the medium containing it can be sterilized (separately from glucose) and stored for at least a week with satisfactory results (8). Medium containing cysteine as reducing agent may be kept not longer than 3 to 4 days.

If the medium is autoclaved for 15 minutes at 15 pounds in the presence of glucose at pH 7.2, caramelization occurs and substances are formed which act as growth inhibitors. Furthermore, under these conditions, the addition of ascorbic acid or cysteine to the medium may fail to initiate growth. The medium containing glucose may be heated 20 minutes in a boiling water bath without producing an appreciable quantity of inhibitors. Also, growth may be obtained in the boiled medium without the addition of a reducing agent.

Table V shows the effect of decreasing the amount of inoculum on the growth obtained in the synthetic medium. The seed cultures which contained  $1.3 \times 10^9$  organisms per cc. (as estimated from the turbidimetric reading of 14.6 microamperes) were washed and serially diluted as described under "Inoculum." The inoculum from the highest dilution, which corresponded to  $3.2 \times 10^{-9}$  cc. of original culture, was calculated to contain four organisms. The growth resulting from inoculation with this smallest inoculum was almost equal after 20 hours incubation to that obtained from large inocula. 15 hours incubation required an inoculum containing between 100 and 500 organisms to produce growth as dense as that from large inocula. The fact that this luxuriant growth was obtained with washed inocula containing only a few organisms seems to justify the conclusion that the synthetic medium contains all the substances required for growth of the organism. Twelve consecutive transfers of *Clostridium perfringens* have been made in this synthetic medium without apparently altering the characteristics of the organism.

TABLE V  
Effect of Dilution of Inoculum on Growth

Tube No.	Quantity of washed cells used for inoculum	No. of cells in inoculum*	Turbidity† Time of incubation at 45°	
			15 hrs.	20 hrs.
	cc.			
1	$5 \times 10^{-4}$	$6.5 \times 10^8$	17.0	17.5
2			19.0	19.0
3	$10^{-4}$	$1.3 \times 10^8$	17.5	18.0
4			18.5	18.5
5	$10^{-5}$	$1.3 \times 10^7$	17.5	18.0
6			18.0	18.0
7	$2 \times 10^{-6}$	$2.6 \times 10^6$	17.2	18.0
8			18.0	18.5
9	$4 \times 10^{-7}$	$5.2 \times 10^5$	17.5	18.5
10			16.5	18.0
11	$8 \times 10^{-8}$	$10^5$	16.0	18.0
12			14.5	17.5
13	$1.6 \times 10^{-8}$	$2 \times 10^4$	14.0	17.0
14			15.0	17.5
15	$3.2 \times 10^{-9}$	4	13.0	16.2
16			12.5	15.0

\* Calculated on the basis that the original seed culture contains  $1.3 \times 10^9$  cells per cc.

† Microamperes.

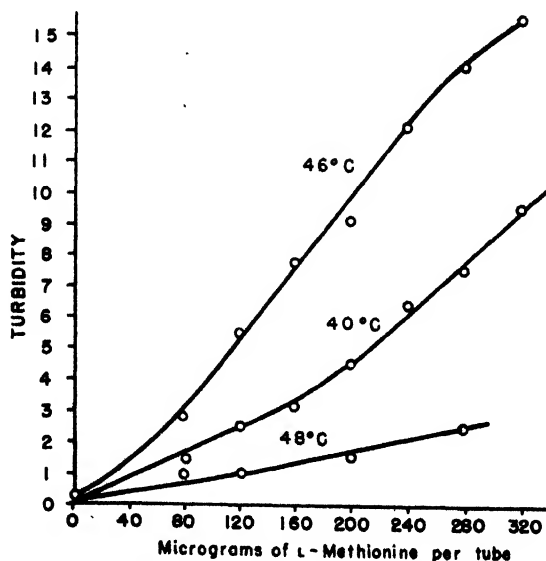


FIG. 5. Effect of temperature on the growth of *Clostridium perfringens* for a period of 16 hours.

Fig. 5 shows the effect of temperature on the growth of *Clostridium perfringens*. It is evident from these experiments that growth is most rapid at 46°. Above 48° almost no growth takes place. Because the growth at 43° is only slightly less rapid than that at 46°, temperatures in this range are suitably employed when the organism is used for assay purposes.

#### SUMMARY

1. The organism, *Clostridium perfringens* BP6K, may be grown luxuriantly in a chemically defined medium consisting of known amino acids, adenine and uracil, vitamins (biotin, calcium *d*-pantothenate, pyridoxamine or pyridoxal, and riboflavin), salts ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , and  $\text{NaCl}$ ), glucose, phosphate buffer, and ascorbic acid.

2. A satisfactory stock culture medium and a seed culture medium have been devised for this organism.

3. Pyridoxamine or pyridoxal (or large quantities of pyridoxine) eliminate the requirements of *Clostridium perfringens* for lysine, alanine, aspartic acid, and glycine.

4. The addition of adenine and uracil reduces the induction period of growth of the organism.

5. The optimum temperature range for rapid growth is 43–46°.

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# A MICROBIOLOGICAL PROCEDURE FOR THE ASSAY OF AMINO ACIDS WITH *CLOSTRIDIUM PERFRINGENS* (WELCHII) BP6K\*

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In the previous paper on the growth requirements of *Clostridium perfringens* BP6K it was shown that thirteen amino acids, *i.e.* arginine, leucine, histidine, isoleucine, methionine, threonine, phenylalanine, tryptophan, valine, glutamic acid, serine, cystine, and tyrosine, are essential for the growth of the organism. The omission of any one of these amino acids from an otherwise complete medium resulted in either very little or no growth. The possibility of using this rapidly growing organism for amino acid assay has been investigated and the results indicate that *Clostridium perfringens* may be used satisfactorily for the assay of the above amino acids.

This procedure has a number of advantages over the present microbial assay methods for determining amino acids: (a) The method does not require aseptic technique. (b) The assay is rapid. The induction period is so short that growth is visible in 2 to 3 hours after inoculation and the assay is completed in 16 hours. (c) The same seed culture may be used for 1 week as a source of the inoculum. (d) The response of the organism to increments of the amino acids essential for the rat is more nearly linear than with other organisms. (e) The culture medium is not autoclaved, and possible destruction of any of the constituents is thereby avoided.

## EXPERIMENTAL

The procedures for the preparation of the "stock culture" and "seed culture" media, transfer, and growth of *Clostridium perfringens* on these media, and the preparation of the double strength basal medium for assay purposes have been given in the previous paper.

**Inoculum**—1 cc. of the seed culture obtained aseptically from the stock seed culture is centrifuged and the supernatant discarded. The packed cells are resuspended in 10 cc. of distilled water and centrifuged a second time. These washed cells<sup>1</sup> are resuspended in 1 cc. of water and a dilution

\* The work described in this paper was assisted by the United States Army.

<sup>1</sup> An unwashed cell suspension of 1:200 was used satisfactorily for the assay of histidine, leucine, isoleucine, arginine, phenylalanine, and tyrosine.



of 1:200 is made with water. 1 drop (0.05 cc.) of the diluted cell suspension is used for the inoculation of each tube of medium.

*Procedure for Assay*—The procedure for assaying an amino acid in this chemically defined medium is as follows:

A set of eight to ten Pyrex culture tubes (15 × 150 mm.) is used to obtain a standard growth curve. 5 cc. of double strength basal medium (deficient in the amino acid being assayed) are pipetted into each tube. Graded amounts of the amino acid under test are added to each tube. The final volume of each tube is adjusted to 10 cc. with distilled water. The amount of the amino acid to be added per tube to obtain a complete growth curve varies with each particular amino acid to be assayed. For instance, the standard curve for tryptophan requires a range of 0 to 80  $\gamma$  per tube; therefore, eight different levels of the amino acid, *i.e.* 0, 10, 20, etc., up to 80  $\gamma$ , are added per tube. However, the range of the standard curve for each amino acid is different, as is shown in Figs. 1 to 6.

To assay the sample for an amino acid several different, accurately measured quantities of the diluted hydrolysate, adjusted to pH 7.1 to 7.2, are added in duplicate to 5 cc. quantities of the basal medium (deficient in the amino acid to be assayed). At least two of the dilutions should correspond to points near the middle of the standard growth curve. The final volume is made to 10 cc. with distilled water.

0.1 cc. of sodium azide solution containing 2 mg. per cc. is added to all tubes. The contents of each tube are mixed by inversion. Glass caps are used to cover the tubes. The tubes are placed in a boiling water bath for 20 minutes, cooled, and inoculated with 1 drop of the diluted inoculum, after which they are incubated in a constant temperature water bath at 45° for a period of 16 hours.

After incubation the contents of the tubes are mixed by inversion and the density of growth determined with a turbidimeter (1). The optical density may be determined with an Evelyn photoelectric colorimeter equipped with a 660 m $\mu$  filter.

*Preparation of Sample for Analysis*—The procedure of Stokes *et al.* (2) is used for preparing a sample of protein for analysis. 1 gm. of protein is introduced into a small Pyrex tube, 10 cc. of 3.5 N HCl are added, and the tube is sealed. The sealed tube is autoclaved 10 hours at 15 pounds pressure, cooled, and the contents of the tube are carefully washed into a graduated cylinder. NaOH is added until the point of neutralization is reached, and the final volume is adjusted to 300 cc.

Tryptophan analysis is made on an alkaline hydrolysate of the protein. 50 mg. of the dried protein are hydrolyzed with 4 cc. of 5 N NaOH in sealed tubes for 10 hours at 15 pounds pressure. The precipitate that forms as a result of the hydrolysis is filtered off and washed with distilled water.

The combined washings are added to the filtrate. The pH of the filtrate is adjusted to 7.2 and the final volume made to 50 cc. Complete racemization of the tryptophan is assumed to occur when the sample has been hydrolyzed under these conditions. Since D-tryptophan is inactive for *Clostridium perfringens*, the final results of the assay must be multiplied by a factor of 2 to obtain the correct tryptophan value.

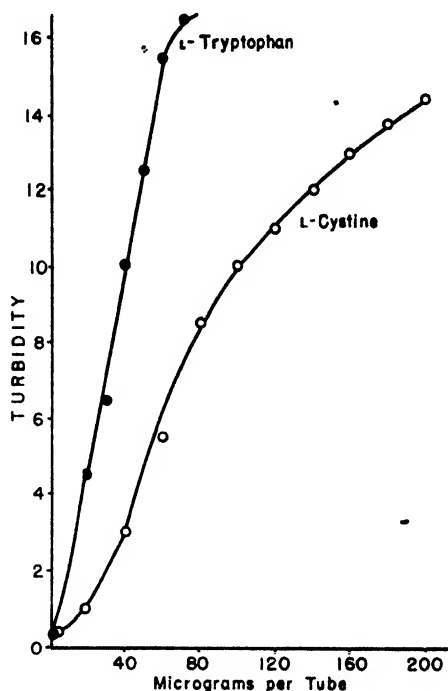


FIG. 1. Standard growth curves of L-tryptophan and L-cystine

#### RESULTS AND DISCUSSION

Figs. 1 to 6 show the standard curves for L-methionine, L-tryptophan, DL-threonine, DL-phenylalanine, L-histidine, L-tyrosine, L-leucine, DL-valine, L-arginine, DL-isoleucine, L-glutamic acid, DL-serine, and L-cystine. The curves show a linear response to increments of the amino acids essential for the rat, while serine, cystine, and tyrosine give sigmoid growth curves. All the indispensable amino acids with the exception of lysine may be assayed. However, lysine may be assayed provided the sample tested contains neither pyridoxamine nor pyridoxal. Fig. 7 shows a standard growth curve for lysine in the presence of 60  $\gamma$  of pyridoxine per tube.

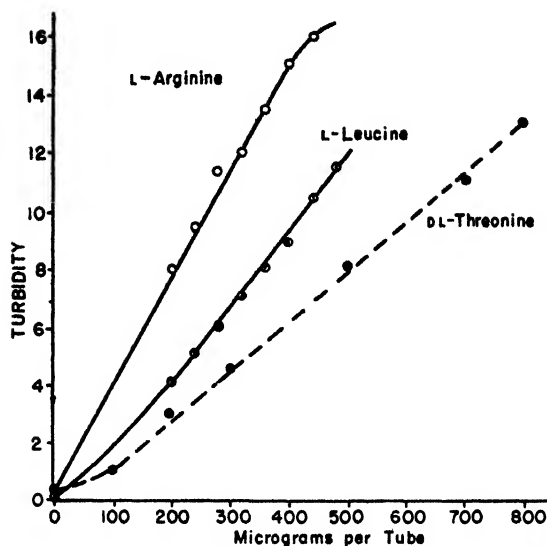


FIG. 2. Standard growth curves of L-arginine, L-leucine, and DL-threonine

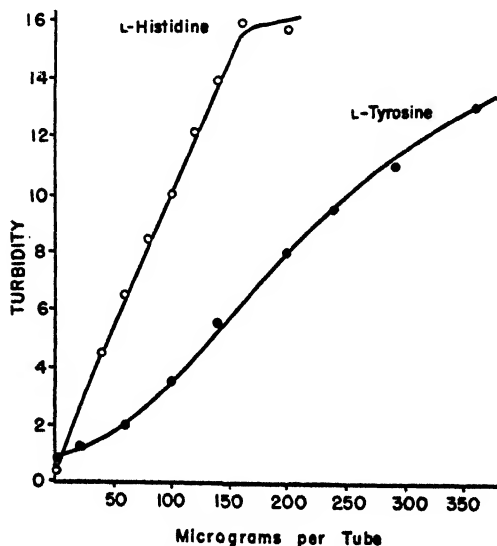


FIG. 3. Standard growth curves of L-histidine and L-tyrosine

The correlation of cell counts, turbidities, and optical densities is shown in Fig. 8. The cell counts were made according to the United States Army method (3). Turbidities were determined with a photoelectric turbidimeter and represent galvanometer readings; *i.e.*, microamperes. Optical

densities were calculated from the transmission values obtained with an Evelyn photoelectric colorimeter (optical density =  $2 - \log G$ ) with a 660

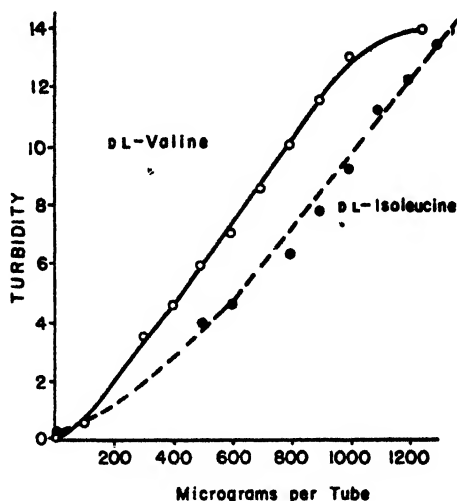


FIG. 4. Standard growth curves of DL-valine and DL-isoleucine

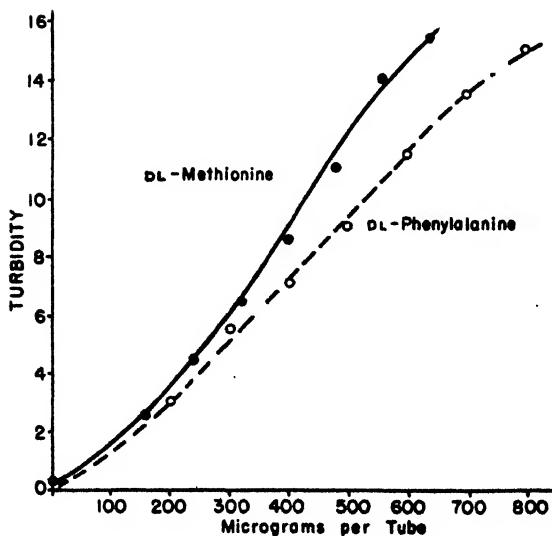


FIG. 5. Standard growth curves of DL-methionine and DL-phenylalanine

m $\mu$  filter. The measurements with the Evelyn photoelectric colorimeter were made on the undiluted culture tubes. The results obtained indicate that turbidity and optical density are proportional to the cell count.

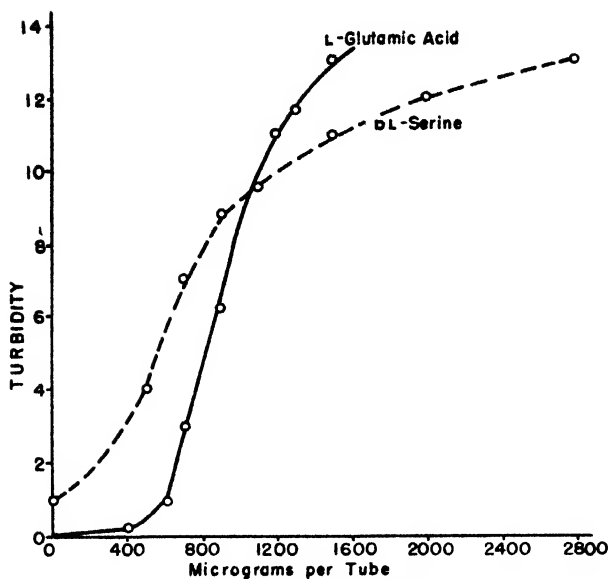


FIG. 6. Standard growth curves of L-glutamic acid and DL-serine

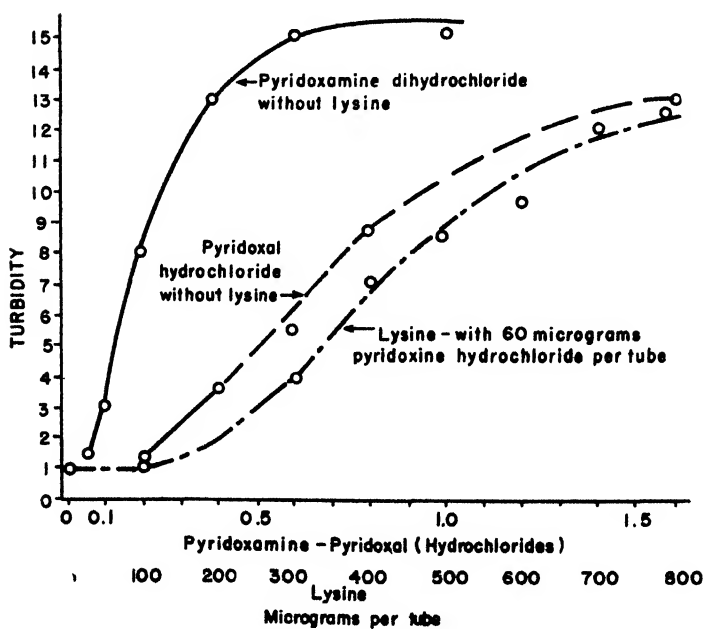


FIG. 7. Response of *Clostridium perfringens* to pyridoxamine and pyridoxal on a medium without lysine, and the growth response for L-lysine in the presence of pyridoxine.

Table I shows the amino acid analyses of  $\beta$ -lactoglobulin, egg albumin, and silk fibroin as determined with *Clostridium perfringens* in this laboratory, and, for comparison, the results of Stokes *et al.*, determined with *Streptococcus faecalis* on samples of the same preparation of protein. The protein samples were dried and hydrolyzed under conditions described by Stokes *et al.* (2). The analytical results obtained by these two different organisms are essentially in agreement, except that the phenylalanine content of  $\beta$ -lactoglobulin was found to be 3.2 instead of 4.3 per cent

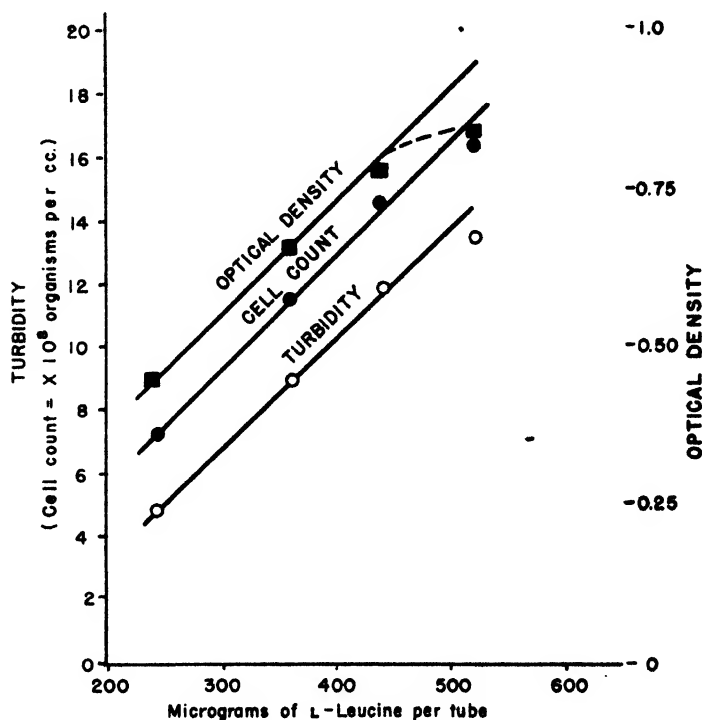


FIG. 8. Comparison of cell counts, turbidities, and optical densities

However, our result is in close agreement with that of Brand *et al.*, who have reported the phenylalanine content of  $\beta$ -lactoglobulin to be 3.5 per cent.

The use of non-aseptic technique in making the assay is an advantageous departure from the usual microbiological assay procedure. Sterilization of the medium in a boiling water bath instead of autoclaving reduces the possibility of destruction of vitamins and amino acids and the formation of inhibitors. Snell (5) has shown that autoclaving pyridoxal with tryptophan or histidine destroys the growth-promoting activity of this vitamin

TABLE I

*Amino Acid Content of Purified Proteins*

The proteins were dried at 105° for 3 hours. The results were calculated as per cent of dry weight.

		Histidine	Arginine	Leucine	Isoleucine	Valine	Methionine	Threonine	Tryptophan	Phenylalanine
$\beta$ -Lacto-globulin	<i>Clostridium perfringens</i>	1.6		15.1	7.3	5.5	2.6	4.8		3.2
	<i>Streptococcus faecalis</i> *	1.5	2.8	15.3	7.0	5.5	2.5	4.6	2.1	4.3
Egg albumin	<i>Clostridium perfringens</i>	2.4	5.9	8.5	7.8	7.5	4.6	3.7	1.4	7.5
	<i>Streptococcus faecalis</i>	2.3	5.9	9.2	7.0	7.0	4.1	3.6	1.4	7.9
Silk fibroin	<i>Clostridium perfringens</i>	0.36	0.98			3.45		1.3	0.34	
	<i>Streptococcus faecalis</i>	0.4	1.11	0.93	1.15	3.5	0.15	1.36	0.44	1.49

\* Stokes, Gunness, Dwyer, and Caswell analyzed samples of the same preparation of protein, using *Streptococcus faecalis*.

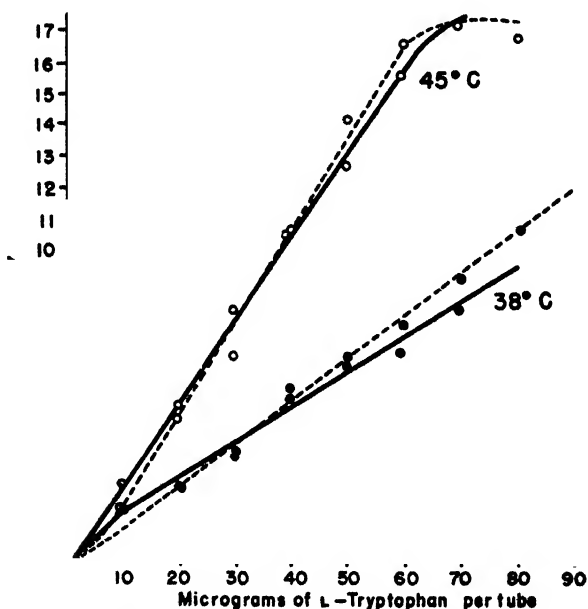


FIG. 9. Standard growth curves of L-tryptophan. Broken lines, growth obtained under aseptic conditions; solid lines, growth obtained under non-aseptic conditions with sodium azide present in the medium.

for certain organisms. Not only is there a considerable saving of time in performing the assay, but the assay may be made by semiskilled personnel. Standard growth curves obtained by the non-aseptic technique have been checked against growth curves obtained under aseptic conditions.

Fig. 9 shows the similarity of standard growth curves for tryptophan obtained by using aseptic and non-aseptic conditions. Under aseptic conditions the tubes of medium were sterilized for 10 minutes at 15 pounds pressure, 0.4 cc. of sterile 25 per cent glucose was added, and the tubes were inoculated with a washed diluted cell suspension. Under non-aseptic conditions the tubes of medium containing glucose and sodium azide were boiled 20 minutes, cooled, and inoculated with a diluted cell suspension which was previously washed under non-aseptic conditions.

Non-aseptic technique in making assays may be employed without running the risk of contamination for the following reasons: (a) Heating the assay tubes in a boiling water bath for 20 minutes destroys most of the vegetative bacteria; (b) the use of sodium azide (6) inhibits the growth of most aerobes; (c) the short induction period reduces the possibility of contamination; and (d) the high incubation temperature of 45° inhibits the growth of most air-borne organisms.

The authors are indebted to Alice Griner Tytell for making the cell counts; to Dr. W. H. Stein of the Rockefeller Institute for  $\beta$ -lactoglobulin, egg albumin, and silk fibroin; and to Dr. J. S. Fruton for the D and L isomers of methionine.

#### SUMMARY

A microbiological assay procedure has been devised for the determination of thirteen amino acids, nine of which are essential for the rat. The advantages of this procedure are as follows:

1. The assay may be done under non-aseptic conditions.
2. The organism grows so rapidly that the assay is completed in 16 hours.
3. One seed culture may be used for at least 1 week.
4. The culture medium need not be sterilized by autoclaving.
5. The response of the organism to increments of the amino acids essential for the rat is very nearly linear.

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3. Simmons, J. S., and Gentzkow, C. J., *Laboratory methods of the United States Army*, Philadelphia, 5th edition, 404 (1944).
4. Brand, E., Saidel, L. J., Goldwater, W. H., Kassell, B., and Ryan, F. J., *J. Am. Chem. Soc.*, **67**, 1524 (1945).
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## LETTERS TO THE EDITORS

### THE LIMITATIONS OF INHIBITION ANALYSIS

Sirs:

A method for the identification of the products of enzyme systems of intact cells by means of inhibition studies has been described by Shive and associates under the term "inhibition analysis."<sup>1, 2</sup> They have assumed

#### *Effect of 5-Bromouracil on Toxicity of 5-Nitrouracil*

Pteroylglutamic acid <i>mg. per 10 ml.</i>	Nitrouracil <i>mg. per 10 ml.</i>	Titer, ml. 0.1 N per 10 ml.	
		Without bromouracil	Bromouracil, 100 $\gamma$ per 10 ml.
0.22	0.0	4.6	3.65
	0.16	0.8	
	0.40	0.6	
	1.0	0.6	1.95
	2.5	0.6	1.3
	6.25	0.8	1.2
1.10	0.0	10.9	10.45
	0.16	2.7	
	0.40	1.2	
	1.0	0.6	6.45
	2.5	0.8	4.2
	6.25	0.7	2.6
5.50	0.0	14.4	14.5
	0.16	6.5	
	0.40	4.3	
	1.0	1.1	13.2
	2.5	0.8	10.1
	6.25	1.7	6.5
Inhibition ratio.....		ca. $7 \times 10^5$	ca. $3 \times 10^7$

that any substance which restores growth to a bacterial culture which is inhibited by an antimetabolite can thus be identified as a normal metabolic product. In particular, if, on the addition of the substance, the inhibitor becomes ineffective or effective only at a higher concentration, the sub-

<sup>1</sup> Shive, W., and Macow, J., *J. Biol. Chem.*, **162**, 451 (1946).

<sup>2</sup> Beerstecher, E., Jr., and Shive, W., *J. Biol. Chem.*, **164**, 53 (1946).

stance is assumed to be a product of the enzyme system with which the antimetabolite interferes.<sup>2-4</sup>

In extensive studies of the effects of pyrimidine derivatives on *Lactobacillus casei* in these laboratories a considerable number of observations have accumulated which cannot be reconciled with this theory. At times "inhibition analysis" suggests conclusions which obviously are false. The following example will clarify this point.

In the table are given the results of experiments with 5-nitrouracil and 5-bromouracil on the growth of *L. casei*.<sup>5</sup> The data were selected from more extensive experiments which will be reported in detail later but are representative of several similar studies. It will be seen that 5-nitrouracil inhibits the growth of *L. casei* competitively with pteroylglutamic acid (PGA), giving a constant inhibition ratio of  $7 \times 10^5$ . In the presence of bromouracil, however, the inhibitory effect of nitrouracil is reversed; in the example chosen, the inhibition ratio is increased by a factor of about 50 and becomes larger with increasing bromouracil concentrations.

Whatever the explanation of these observations, it is obvious that bromouracil is not the product of the PGA enzyme system which "inhibition analysis" indicates it to be. This illustrates the type of fallacy which may result from a too facile interpretation of metabolite-antimetabolite studies. It may very well be true that when the product of a known enzyme system can be supplied the system can be by-passed for growth purposes. It does not follow that every substance which relieves an inhibition of a microorganism can be assumed to be a product, or substrate, of normal metabolism.

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<sup>2</sup> Beerstecher, E., Jr., and Shive, W., *J. Biol. Chem.*, **167**, 49 (1947).

<sup>4</sup> Rogers, L. L., and Shive, W., *J. Biol. Chem.*, **172**, 751 (1948).

<sup>5</sup> Hitchings, G. H., Falco, E. A., and Sherwood, M. B., *Science*, **102**, 251 (1945).

## TWO NEW REACTIONS PRODUCING ACETYL PHOSPHATE\*

Sirs:

During a study of the conversion of ethanol and acetate to caproate by an enzyme preparation obtained from *Clostridium kluyveri*<sup>1</sup> two new reactions producing acetyl phosphate have been observed.

TABLE I

23 mg. of dry cell-free enzyme preparation in 2.0 ml. of 0.04 M veronal buffer, pH 8.1, containing 0.001 M methylene blue and the indicated amounts of inorganic phosphate (P<sub>i</sub>) and organic substrates were shaken in air at 26°.

Substrate	P <sub>i</sub> initial	Incubation time	ΔP <sub>i</sub> *	ΔP <sub>ao</sub> †	-O <sub>2</sub>	Δ acetate + acetyl phosphate	$\frac{\Delta P_{ao}}{-O_2}$	$\frac{\Delta P_{ao}}{\Delta P_i}$
	micro-moles	min.	micro-moles	micro-moles	micro-moles	micromoles		
CH <sub>3</sub> CHO								
150 micromoles	21	120	-17.8	21.4	10.7	18.0	2.00	1.20
	151	440	-136	138	66.2	138	2.10	0.99
C <sub>2</sub> H <sub>5</sub> OH								
500 micromoles	206	640	-112	107	133	137	0.78	1.05

\* Method of Lipmann and Tuttle (Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **153**, 571 (1944)).

† Method of Lipmann and Tuttle (*J. Biol. Chem.*, **159**, 21 (1945)).

TABLE II

85 mg. of dry cell-free enzyme preparation in 5.0 ml. of 0.021 M phosphate buffer, pH 5.7, and the indicated amount of acetoacetate were incubated *in vacuo* in Thunberg tubes for 4 hours at 26°.

Acetoacetate, initial	Δ acetoacetate*	Δ acetate	Δ P <sub>i</sub>	Δ P <sub>ao</sub>	$\frac{\Delta P_{ao}}{\Delta P_i}$	$\frac{\Delta \text{acetate}}{\Delta \text{acetoacetate}}$
micromoles	micromoles	micromoles	micromoles	micromoles		
175	61.5	115	-30.5	32.5	1.06	1.87
337	72.5	150	-38.0	40.5	1.06	2.07

\* Method of Jowett and Quastel (Jowett, M., and Quastel, J. H., *Biochem. J.*, **29**, 2145 (1935)).

The first reaction is an oxidation of acetaldehyde in the presence of inorganic phosphate. In the absence of phosphate or arsenate, acetaldehyde

\* This work was supported in part by a research grant from the United States Public Health Service.

<sup>1</sup> Bornstein, B. T., and Barker, H. A., *J. Biol. Chem.*, **172**, 659 (1948).

is not oxidized. The oxidation can be carried out aerobically (Equation 1) with methylene blue as an electron carrier, or anaerobically, without methylene blue.



Under the latter conditions acetaldehyde apparently undergoes a dismutation. During the oxidation of acetaldehyde, inorganic phosphate disappears and an almost equivalent quantity of a labile phosphate compound is formed (see Table I).

The second reaction may be described as a phosphoroclastic decomposition of acetoacetate (Equation 2).



The data of Table II show that for each mole of acetoacetate decomposed 2 moles of acetate (free acetate + acetyl phosphate) are formed. Inorganic phosphate disappears and an equivalent amount of acyl phosphate is formed.

The amount of free acetate formed is considerably greater than that predicted by Equation 2; the excess acetate is probably due to a secondary hydrolysis of a part of the acetyl phosphate formed.

The labile phosphate compound formed in both of the above reactions combines with hydroxylamine to give a hydroxamic acid under the conditions used by Lipmann and Tuttle. It is completely hydrolyzed in less than 10 minutes at room temperature by the Fiske-Subbarow phosphate reagent, and the rate of hydrolysis in 0.5 N HCl is identical with that of synthetic monoacetyl phosphate.

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## ISOLATION OF A PEPTIDE IN GUINEA PIG LIVER HOMOGENATE AND ITS TURNOVER OF LEUCINE\*

*Sirs:*

Leucine was synthesized with  $C^{14}$  in the carboxyl group. 10 mg. of the radioactive amino acid (DL) and 0.66 gm. (wet weight) of guinea pig liver homogenate were added to a reaction mixture containing 1.3 per cent of an amino acid mixture corresponding to the composition of casein and 0.005 M fumarate, all in a final volume of 4 ml. of isotonic saline solution<sup>1</sup> at pH 7.4. The reaction was carried out under oxygen for 6 hours at 38°.

After precipitation of the proteins by boiling at pH 5.0, the non-protein filtrate was chromatographed on starch with a solvent mixture consisting of 1 part 0.1 N HCl, 2 parts of N-propanol, and 1 part of N-butanol according to the method of Moore and Stein.<sup>2</sup> A radioactive fraction was found which emerged from the column before the leucine. This fraction was concentrated and purified by chromatography on a smaller starch column. The distribution of the color on treatment with ninhydrin and the radioactivity gave a single zone of coinciding ninhydrin color and radioactivity.

The fraction was hydrolyzed with boiling 20 per cent hydrochloric acid for 24 hours, the hydrolysate chromatographed on starch, the fractions analyzed with ninhydrin, and their radioactivity determined as before.

Whereas before hydrolysis only one ninhydrin zone was obtained, after hydrolysis there were many zones. All the radioactivity was in the leucine fraction.

Once the point of emergence of the peptide had been determined by its radioactivity, the non-protein filtrate of guinea pig liver (prepared without prior incubation or any additions) was chromatographed. A peptide was found which emerged in the same position as did that from the incubated digest.

The identity of the amino acids giving the different ninhydrin zones after hydrolysis was determined by chromatography on filter paper<sup>3</sup> with known amino acids as references. The presence of the following was established:

\* This work is part of that done under contract with the Office of Naval Research, United States Navy Department.

The  $C^{14}$  used in this investigation was supplied by the Monsanto Chemical Company, Clinton Laboratories, and was obtained on allocation from the United States Atomic Energy Commission.

<sup>1</sup> Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **171**, 363 (1947).

<sup>2</sup> Moore, S., and Stein, W. H., *Federation Proc.*, **7**, 174 (1948). Stein, W. H., and Moore, S., *Federation Proc.*, **7**, 192 (1948).

<sup>3</sup> Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38**, 224 (1944).

alanine, aspartic acid, glutamic acid, glycine, leucine, methionine, phenylalanine, and proline. Other amino acids are present; complete amino acid analysis awaits collection of a sufficient amount of the peptide.

The leucine "turnover," estimated from the radioactivity of the leucine obtained after hydrolysis of the peptide, was 40 per cent in 6 hours.

We have obtained evidence by methods similar to the above of other peptides in guinea pig liver.

The authors are indebted to and wish to thank Dr. S. Moore and Dr. W. H. Stein of The Rockefeller Institute for Medical Research for their generosity and courtesy in giving the details of their method of starch chromatography and ninhydrin colorimetry prior to publication.

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# THE INCORPORATION OF ADENINE INTO PENTOSE AND DESOXY-PENTOSE NUCLEIC ACIDS\*

Sirs:

We have carried out an experiment in which labeled adenine<sup>1</sup> was fed and the pentose nucleic acids, PNA, (chiefly cytoplasmic) and the desoxy-pentose nucleic acids, DNA, (nuclear) were separated. The adenine was fed to five adult male rats at a level of 27 mg. per kilo of body weight per day for 10 days. The separation of the two types of nucleic acids from the total viscera was carried out by an application of the Schmidt-Thannhauser technique.<sup>2</sup> Each nucleic acid fraction was shown<sup>3</sup> to be free of the other, within experimental error ( $\pm 5$  per cent). The purines were isolated from

	N calculated	From DNA fraction			From PNA fraction		
		N found	Atom per cent excess N <sup>15</sup>	Calculated on basis of 100 per cent N <sup>15</sup> in adenine fed	N found	Atom per cent excess N <sup>15</sup>	Calculated on basis of 100 per cent N <sup>15</sup> in adenine fed
Adenine (dietary).....			6.29	100		6.29	100
“ picate·H <sub>2</sub> O.....	29.2	28.9	0.024		29.4	0.634	
“ (from picate)....			0.035	0.55		1.00	15.9
Guanine·H <sub>2</sub> SO <sub>4</sub> ·H <sub>2</sub> O.....	33.4	*	0.024	0.32	34.2	0.572	9.1

\* Because of the small sample obtained, this was characterized by counter-current distribution only, and the isotope analysis was performed on a diluted sample.

each fraction and, by counter-current distribution,<sup>4</sup> each was shown to be free of the other.

Analyses of the adenine and guanine isolated from each fraction (see the table) show that in the PNA fraction 15.9 per cent of the adenine and 9.1 per cent of the guanine originated from the dietary adenine. In the purines of the DNA fraction, the low isotope concentrations indicate a replacement which is only 3.5 per cent of that obtained in the PNA fraction, or a ratio of PNA purine to DNA purine turnover of 29:1. Because of the uncertainties in the determination of PNA in the presence of large amounts of DNA, it is impossible to say that the N<sup>15</sup> uptake observed in

\* The authors gratefully acknowledge the use of funds from the Office of Naval Research, the James Foundation, Inc., New York, and Lord and Taylor, New York.

<sup>1</sup> Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, I. F., *J. Biol. Chem.*, **172**, 469 (1948).

<sup>2</sup> Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83 (1945).

<sup>3</sup> Schneider, W. C., *J. Biol. Chem.*, **164**, 747 (1946).

<sup>4</sup> Tinker, J. F., and Brown, G. B., *J. Biol. Chem.*, **173**, 585 (1948).



the DNA fraction may not be partially or entirely ascribed to contaminating PNA, and it is even conceivable that there is a fundamental difference in the origin of the purines of PNA and DNA.

The PNA purine to DNA purine ratio of 29:1 (or greater) found for total viscera, after feeding labeled adenine for 10 days, is much higher than any of the comparable figures on phosphorus turnover, which range from 1.66:1 to 7:1,<sup>5</sup> and emphasizes the extremely slow rate of turnover of the nucleic acid purines in the resting nucleus. If, as the available data indicate, the ratios of turnover of PNA purine to DNA purine and PNA P to DNA P are different, at least some portion of the phosphate moieties of a nucleic acid may be exchanged without the purines of the C-N skeleton being affected.

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<sup>5</sup> Brues, A. M., Tracy, M. M., and Cohn, W. E., *J. Biol. Chem.*, **155**, 619 (1944). Hammarsten, E., and Hevesy, G., *Acta physiol. Scand.*, **11**, 335 (1946). Davidson, J. N., and Raymond, W., *Biochem. J.*, **42**, p. xiv (1948).

# ON THE CONVERSION OF PALMITIC ACID LABELED WITH RADIOACTIVE CARBON TO GLUCOSE BY THE ALLOXAN-DIABETIC RAT\*

Sirs:

Although it is now well established that the carbon of lower fatty acids can be incorporated into the glucose molecule,<sup>1-3</sup> such a conversion for the carbon of long chain fatty acids has not hitherto been demonstrated.

Palmitic acid labeled at its 6th carbon<sup>4</sup> was used in this investigation. 50 mg. of this fatty acid dissolved in 1 cc. of corn oil were administered by

C <sup>14</sup> administered as	Rat No.	24 hr. excretion		Per cent of C <sup>14</sup> recovered		Specific activities* of urine glucose		Specific activity† of expired CO <sub>2</sub>	S.A.‡ CO <sub>2</sub> carbon S.A. glucose carbon
		Urine glucose	Expired CO <sub>2</sub>	Urine glucose	Expired CO <sub>2</sub>	Osazone	Oso-triazole		
		gm.	gm.						
Palmitic acid	1	4.0	12.0	3.9	32.9	123	132	520	4.1
	2	0.9	7.5	2.5	29.7	364	370	754	2.1
	3	7.7	14.9	6.5	23.7	106	108	306	2.9
	4	6.8	13.6	6.7	26.6	126	124	377	3.0
Bicarbonate	5	3.7	15.2	4.4	73	157	152	920	6.0
	6	6.9	17.6	6.9	72	126	134	784	6.0

\* Refers to counts per minute per mg. of glucose carbon of the 24 hour urine sample.

† Refers to counts per minute per mg. of CO<sub>2</sub> carbon of the 24 hour CO<sub>2</sub> sample.

‡ Specific activity.

stomach tube to alloxan-diabetic rats and the urine of these animals collected for the next 24 hours. The rats had access to food. The urinary glucose was isolated as the osazone, which was then converted to the osotriazole derivative. The results obtained with four rats are recorded in the table. The purity of the osazones and osotriazoles was established by the determination of nitrogen content, melting point, and the demonstration of identical specific activities for these two compounds (see the table).

Since it has been shown that the carbon of CO<sub>2</sub> can be incorporated into glucose,<sup>1, 2</sup> the excretion of radioactive glucose by fed alloxan-diabetic rats

\* Aided by a grant from the American Cancer Society (recommended by the Committee on Growth of the National Research Council).

<sup>1</sup> Buchanan, J. M., and Hastings, A. B., *Physiol. Rev.*, **26**, 120 (1946).

<sup>2</sup> Wood, H. G., *Physiol. Rev.*, **26**, 198 (1946).

<sup>3</sup> Bloch, K., *Physiol. Rev.*, **27**, 574 (1947).

<sup>4</sup> Dauben, W. G., *J. Am. Chem. Soc.*, in press.

injected with  $\text{NaHC}^{14}\text{O}_2$  was also investigated. 1 cc. of isotonic saline containing 0.028 mm of  $\text{NaHC}^{14}\text{O}_2$  was injected intraperitoneally into each of two alloxan-diabetic rats. The results of this experiment are recorded in the table.

The absolute amounts of radioactivity found in urinary glucose were small in both experiments. In order to assess the rôle of  $\text{CO}_2$  in the conversion of palmitic acid to glucose, the values for the ratio of specific activity of  $\text{CO}_2$  carbon to specific activity of glucose carbon obtained in the two experiments were compared.

The ratio for the  $\text{CO}_2$  fixation experiment was 6.0. This indicates that 1 of every 6 atoms, or about 16 per cent of the glucose carbon excreted by the alloxan-diabetic rat, was derived from bicarbonate carbon. This value is in good agreement with those reported by Solomon *et al.* for the fixation of carbon dioxide in glycogen by the normal rat.<sup>5</sup>

The fact that the values for this ratio in the palmitic acid experiment were much lower than 6 is of particular significance. This suggests that a process other than carbon dioxide fixation is involved in the incorporation of the 6th carbon of palmitic acid in glucose.

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<sup>5</sup> Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **140**, 171 (1941).

## ACTIVITY OF CRYSTALLINE VITAMIN B<sub>12</sub> FOR CHICK GROWTH

*Sirs:*

Crystalline vitamin B<sub>12</sub>,<sup>1</sup> when added to purified basal diets containing 40 to 70 per cent soy bean meal as the sole source of protein, has been found to exhibit "animal protein factor" activity<sup>2</sup> in chicks obtained from hens fed an all vegetable protein ration.<sup>3</sup> The diets used were inadequate in the unidentified chick growth factor (or factors), described by other investi-

Experiment No.	Basal diet	Supplement	No. of chicks	Weight at 17 days
				gm.
1	40% soy bean meal	None	7, 7	113, 117
		0.2% Wilson's 1:20	7	130
		2% Wilson's L	7	131
		0.000003% vitamin B <sub>12</sub>	7	133
2	70% " " "	None	7	84
		0.3% Wilson's 1:20	7	101
		0.000003% vitamin B <sub>12</sub>	7	116
		0.000006% vitamin B <sub>12</sub>	7	116
3	20% casein	None	7, 7	98, 99
		0.2% Wilson's 1:20	7	123
		2% Wilson's L	7	110
		0.000003% vitamin B <sub>12</sub>	7	127
4	20% "	0.000006% vitamin B <sub>12</sub>	7	116
		None	7	100
		0.2% Wilson's 1:20	7	118
		2% condensed fish solubles	7	125
		0.0000015% vitamin B <sub>12</sub>	7	146

gators<sup>4</sup> since reportedly good crude sources of the animal protein factor promoted appreciable increases in growth when fed as supplements to these basal diets.

The percentage composition of the 40 per cent soy bean meal basal diet was as follows: soy bean meal 40, cerelese 37.9, salt mixture 6,<sup>5</sup> Ruffex 5.

<sup>1</sup> Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, **107**, 396 (1948).

<sup>2</sup> Scott, M. L., Norris, L. C., and Heuser, G. F., *J. Biol. Chem.*, **167**, 261 (1947).

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wheat germ oil 4.5, calcium gluconate 2.5, glycine 2, DL-methionine 0.9, L-arginine 0.5, L-cystine 0.2, choline 0.2, AD powder (5000 A, 1000 D) 0.1, inositol 0.1, *p*-aminobenzoic acid 0.03, niacin 0.01, calcium pantothenate 0.004, riboflavin 0.002, thiamine 0.002, pyridoxine 0.002, pteroylglutamic acid 0.004, menadione 0.0004, and biotin 0.00004. The 70 per cent soy bean meal diet contained 70 per cent soy bean meal, 7.9 per cent cerelose, and the remaining ingredients as listed above. The 20 per cent casein diet contained 20 per cent vitamin-free casein (Labco), and an additional 20.9 per cent cerelose in place of the soy bean meal and methionine stated above.

Typical growth data for day-old female white Leghorn chicks given the basal diet with or without supplements of crystalline vitamin B<sub>12</sub>, or various crude sources of the animal protein factor, are shown in the table.

It is apparent that quantities of crystalline vitamin B<sub>12</sub> as small as 6  $\gamma$  per kilo of diet stimulated growth of chicks fed diets low in the animal protein factor. Under these conditions the optimal growth requirement appeared to be less than 30  $\gamma$  per kilo of diet.

Since the crystalline substance elicited growth responses comparable to those obtained by supplementation with crude sources of the animal protein factor, it is possible that vitamin B<sub>12</sub> is identical with or closely related to this factor.

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# INFLUENCE OF ADRENALECTOMY AND FASTING ON THE INCORPORATION OF ISOTOPIC NITROGEN INTO THE TISSUES OF MICE\*

*Sirs:*

Previous experiments suggested<sup>1</sup> that the growth of lymphoid structures of fasted, adrenalectomized animals resulted from immobility of lymphoid tissue nitrogen and translocation of nitrogen from other tissues. Experiments to test this hypothesis have been initiated with the aid of N<sup>15</sup>.

20 gm. CBA male mice were employed. Adrenalectomized mice were used 48 hours postoperatively. All mice received 90 mg. (in 1.2 ml. of

Tissue	Atom per cent excess of N <sup>15</sup> 18 hrs. following last injection of isotope			Per cent difference in N <sup>15</sup> in adrenalectomized, fasted vs. intact, fasted mice
	Intact, fed	Intact, fasted 66 hrs.	Adrenalectomized, fasted 66 hrs.	
Liver	0.417	0.785	0.803	+2
Gut	0.439	0.868	0.954	+10
Kidney		0.513	0.553	+8
Spleen	0.325	0.397	0.596	+50
Thymus and lymph nodes		0.470	0.706	+50
Serum proteins	0.396	0.941	1.347	+44
Muscle		0.055	0.094	+50

H<sub>2</sub>O) of labeled glycine (32.0 atom per cent excess N<sup>15</sup>) in six divided, intra-peritoneal injections over a period of 48 hours. Fasting was initiated at the time of the first injection of glycine, with 0.9 per cent sodium chloride available to adrenalectomized mice. Animals were exsanguinated, and selected organs and tissues from four animals were removed and pooled, ground while frozen, and extracted three times with cold 7 per cent trichloroacetic acid. The residues were freed of trichloroacetic acid, fat-extracted, and dried. Total serum proteins were precipitated from pooled sera of four animals by addition of an equal volume of 20 per cent trichloroacetic acid, and the precipitate treated as described above. Some typical data are shown in the table.

\* This investigation was aided by a grant from the Josiah Macy, Jr., Foundation and from the James Hudson Brown Memorial Fund of the Yale University School of Medicine.

<sup>1</sup> White, A., and Dougherty, T. F., *Endocrinology*, **41**, 230 (1947).

It will be seen that the net rate of incorporation of nitrogen, injected as glycine, into tissues is more rapid in fasted than in fed animals. This rate is also more rapid in the spleen, lymphoid tissue, and serum proteins of adrenalectomized than in intact fasted mice. The difference in muscle is of questionable significance because of the low isotope concentration. Moreover, the gastrocnemius muscle used for analysis may not be representative of all muscle. The findings confirm the retardation in nitrogen catabolism reported following adrenalectomy.

The data are also of some interest in relation to the question of the site of synthesis of the serum proteins. The high isotope concentration in the serum proteins of intact, fasted animals, and particularly in the serum proteins of the adrenalectomized, fasted mice, is strikingly higher than that of any other tissue examined. Inasmuch as there is no difference in the isotope content of the livers of the two groups of fasted animals, whereas there is a marked difference in isotope concentration in the serum proteins, it would appear that a portion of the latter must be of extrahepatic origin. The data are in contrast to those of Stoerk, John, and Eisen<sup>2</sup> who found that the rate of synthesis of serum proteins in rats was not affected by adrenalectomy.

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<sup>2</sup> Stoerk, H. C., John, H. M., and Eisen, H. M., *Proc. Soc. Exp. Biol. and Med.*, **66**, 25 (1947).

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## NUCLEOTIDE PYROPHOSPHATASE AND TRIPHOSPHOPYRIDINE NUCLEOTIDE STRUCTURE

Sirs:

DPN pyrophosphatase<sup>1</sup> in washed particles of rabbit kidney hydrolyzes diphosphopyridine nucleotide (DPN) at the pyrophosphate bond. Difficulties in purifying the kidney enzyme prompted the use of potato extracts in which Lowry<sup>2</sup> has found a pyrophosphatase for flavin-adenine dinucleotide (FAD). From crude potato extracts, DPN pyrophosphatase was purified over 600-fold by ammonium sulfate and ethanol fractionation, heating, and adsorption on alumina gel C $\gamma$ .

In the first four columns of the accompanying table, preparations at various stages of purification are compared with respect to pyrophosphatase activity on DPN, FAD, triphosphopyridine nucleotide (TPN), and adenosine triphosphate (ATP). The relative constancy in the ratios of DPN, TPN, and FAD activities suggests a single enzyme. With ATPase, constant ratios appeared only in more purified fractions, the early increase in ratio probably being due to the removal of Kalckar's<sup>3</sup> ATPase. DPN strongly inhibited ATP splitting only in the purified fractions and FAD and TPN splitting in all fractions. The data suggest that the dinucleotide pyrophosphatase also breaks the pyrophosphate bonds of ATP.

The position of the third phosphate in TPN has been studied with the purified pyrophosphatase. The last four columns of the table summarize an experiment in which 6.65 micromoles of TPN were split by incubation with 0.3 mg. of the most purified pyrophosphatase preparation in 2 ml. of 0.025 M glycylglycine buffer, pH 7.40, for 40 minutes at 37°. No adenylic acid<sup>4</sup> or orthophosphate was produced. Orthophosphate liberated by 10 minutes hydrolysis at 100° in 1 N H<sub>2</sub>SO<sub>4</sub> (2.34 micromoles) was essentially unchanged. With lead acetate, a partial separation of the mononucleotide products was obtained. The insoluble fraction contained adenine and phosphate in a ratio of approximately 1:2 and only 7 per cent of the nicotinamide-ribose moiety.<sup>5</sup> The soluble fraction contained phosphate and nicotinamide-ribose moiety in a ratio of approximately 1:1. The acid lability of the phosphate in the insoluble fraction approximated that of a

<sup>1</sup> Kornberg, A., and Lindberg, O., *Federation Proc.*, **7**, 165 (1948).

<sup>2</sup> Personal communication from Dr. O. H. Lowry. I am also grateful to Dr. S. P. Colowick for suggesting potato as a source for DPN pyrophosphatase.

<sup>3</sup> Kalckar, H. M., *J. Biol. Chem.*, **167**, 461 (1947).

<sup>4</sup> Tested with Schmidt's deaminase (Kalckar, H. M., *Science*, **90**, 131 (1944)).

<sup>5</sup> Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **167**, 157 (1947).



mixture of equal parts of adenosine-3- and adenosine-5-phosphate. However, this fraction contained an organic phosphate impurity, the acid lability

Purity of fractions*	Ratios†			Substance estimated	Reaction mixture (before incubation)	Water-insoluble lead salts	Water-soluble lead salts
	FAD	TPN	ATP		micromoles	micromoles	micromoles
1	2.4	1.4	1.3	TPN‡	6.65		
7	2.2	1.8	13	Acid-labile P	2.10	2.48	0.18
46	2.4	1.2	311	Total P	26.20	16.40	6.80
293	2.4	1.2	351	Adenine§	9.20	8.41	
600	2.4	1.0	335	NMN§			7.15
				Nicotinamide-ribose		0.49	6.53

\* Based on DPN pyrophosphatase activity expressed in arbitrary units.

† Ratio of DPN pyrophosphatase to FAD, TPN, or ATP pyrophosphatase.

‡ Estimated spectrophotometrically by reduction with isocitric acid and isocitric dehydrogenase (Ochoa, S., *J. Biol. Chem.*, **174**, 133 (1948)). The purity of TPN was 0.65. It appears that an adenine nucleotide (not adenosine-5-phosphate) may be an impurity in this preparation.

§ Ultraviolet absorption spectra of the water-insoluble and water-soluble fractions agreed with those of adenine and nicotinamide mononucleotide (NMN), respectively. The coefficients used were  $16 \times 10^3 \times \text{mole}^{-1}$  for adenine and  $5 \times 10^3 \times \text{mole}^{-1}$  for NMN at 260  $\mu$ . The "adenine" of TPN was calculated by assuming an NMN content of 6.65 micromoles.

of which is unknown. Further study is required to establish which position on the ribose of adenosine is occupied by the third phosphate of TPN.

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# INDEX TO AUTHORS

## A

- Adams, Elijah. See *Roberts, Adams, and White*, 379  
 Appel, Muriel. See *Williamson and Appel*, 769  
 Armstrong, Marvin D., and Binkley, Francis. On the failure of DL-homoserine to support growth on a methionine-deficient diet, 889  
 Avakian, Souren. See *Martin, Avakian, and Moss*, 495

## B

- Babb, Virginia. See *Colio and Babb*, 405  
 Barker, H. A. See *Stadtman and Barker*, 1039  
 Barry, Guy T., Sato, Yoshio, and Craig, Lyman C. Distribution studies. X. Attainment of equilibrium, 209  
 —, —, and —. XII. Purity of crystalline penicillins, 221  
 —. See *Sato, Barry, and Craig*, 217  
 Beiler, J. M., and Martin, Gustav J. Inhibition of hyaluronidase action by derivatives of hesperidin, 31  
 Belinskey, Carol. See *Carter, Belinskey, Clark, Flynn, Lytle, McCasland, and Robbins*, 415  
 Bernheim, Frederick, Bernheim, Mary L. C., and Wilbur, Karl M. The reaction between thiobarbituric acid and the oxidation products of certain lipides, 257  
 Bernheim, Mary L. C. See *Bernheim, Bernheim, and Wilbur*, 257  
 Berson, Bella Z. See *Burch, Lowry, Bessey, and Berson*, 791  
 Bessey, Otto A. See *Burch, Lowry, Bessey, and Berson*, 791  
 Beyer, G. Thannhauser. See *Dounce and Beyer*, 859  
 —. See *Dounce, Rothstein, Beyer, Meier, and Freer*, 361  
 Biely, Jacob. See *Halpern and Biely*, 817

- Binkley, Francis. Birefringence of flow of preparations of myosin, 385  
 —. See *Armstrong and Binkley*, 889  
 Bird, H. R., Rubin, Max, and Groschke, A. C. A chick growth factor in cow manure. VII. Its stability and solubility, 611  
 Bischoff, Fritz, and Pilhorn, Harry R. The state and distribution of steroid hormones in biologic systems. III. Solubilities of testosterone, progesterone, and  $\alpha$ -estradiol in aqueous salt and protein solution and in serum, 663  
 Bodansky, Oscar. The inhibitory effects of DL-alanine, L-glutamic acid, L-lysine, and L-histidine on the activity of intestinal, bone, and kidney phosphatases, 465  
 Borsook, Henry, Deasy, Clara L., Haagen-Smit, A. J., Keighley, Geoffrey, and Lowy, Peter H. Isolation of a peptide in guinea pig liver homogenate and its turnover of leucine, 1041  
 Boyd, M. John, Logan, Milan A., and Tytell, Alfred A. A microbiological procedure for the assay of amino acids with *Clostridium perfringens* (welchii) BP6K, 1027  
 —, —, and —. The growth requirements of *Clostridium perfringens* (welchii) BP6K, 1013  
 Brickson, W. L. See *Hankes, Henderson, Brickson, and Elvehjem*, 873  
 Brown, Betty. See *Daniel, Scott, Heuser, and Norris*, 71  
 Brown, George Bosworth, Petermann, Mary L., and Furst, Sidney S. The incorporation of adenine into pentose and desoxypentose nucleic acids, 1043  
 Bubl, Edward C., and Butts, Joseph S. The utilization of D-tyrosine for growth in the rat, 637

- burgh, Helen B., Lowry, Oliver H., Bessey, Otto A., and Berson, Bella Z. The determination of iron in small volumes of blood serum, 791
- Burris, R. H. See *Olson, Fager, Burris, and Elvehjem*, 319
- Butts, Joseph S. See *Bubl and Butts*, 637

## C

- Carter, Herbert E., Belinskey, Carol, Clark, R. K., Jr., Flynn, Edwin H., Lytle, Betty, McCasland, G. E., and Robbins, Mary. Oxidation of inositol by *Acetobacter suboxydans*, 415
- and Loo, Y. H. Oxidation of hydroxy-L-proline by periodate, 723
- Chaikoff, I. L., Lerner, S. R., Entenman, C., and Dauben, W. G. On the conversion of palmitic acid labeled with radioactive carbon to glucose by the alloxan-diabetic rat, 1045
- See *Wolff and Chaikoff*, 555
- Chandler, Joseph P. See *du Vigneaud, Moyer, and Chandler*, 477
- Chanutin, Alfred. See *Gjessing, Ludewig, and Chanutin*, 683
- Chargaff, Erwin. See *Magasanik and Chargaff*, 173
- Cheldelin, Vernon H. See *King and Cheldelin*, 273
- Clark, R. K., Jr. See *Carter, Belinskey, Clark, Flynn, Lytle, McCasland, and Robbins*, 415
- Cohen, Philip P., and Grisolia, Santiago. The intermediate rôle of carbamyl-L-glutamic acid in citrulline synthesis, 389
- See *McGilvery and Cohen*, 813
- Cohen, Seymour S. The synthesis of bacterial viruses. I. The synthesis of nucleic acid and protein in *Escherichia coli* B infected with T<sub>1</sub><sup>+</sup> bacteriophage, 281
- II. The origin of the phosphorus found in the desoxyribonucleic acids of the T<sub>1</sub> and T<sub>2</sub> bacteriophages, 295
- Cello, Lloyd G., and Babb, Virginia. Study of a new stimulatory growth factor, 405

- Comar, C. L., Davis, George K., and Singer, Leon. The fate of radioactive copper administered to the bovine, 905
- Craig, Lyman C. See *Barry, Sato, and Craig*, 209, 221
- See *Sato, Barry, and Craig*, 217
- Critchfield, Frances H. See *Shaffer and Critchfield*, 489

## D

- Damron, Charlotte M. See *Roe, Mills, Oesterling, and Damron*, 201
- Daniel, Louise J., Scott, M. L., Heuser, G. F., and Norris, L. C. Further studies of unidentified factors required in the nutrition of *Lactobacillus casei*, 71
- Dauben, W. G. See *Chaikoff, Lerner, Entenman, and Dauben*, 1045
- Davis, George K. See *Comar, Davis, and Singer*, 905
- Deasy, Clara L. See *Borsook, Deasy, Haagen-Smit, Keighley, and Lowy*, 1041
- Dorfman, Albert, Ott, Melvin L., and Whitney, Richard. The hyaluronidase inhibitor of human blood, 621
- Dounce, Alexander L., and Beyer, G. Thannhauser. The arginase activity of isolated cell nuclei, 859
- , Rothstein, Aser, Beyer, G. Thannhauser, Meier, Rebecca, and Freer, Richard M. A detailed procedure for the preparation of highly purified adenosine triphosphate, 361

## E

- Elion, Gertrude B. See *Hitchings, Elion, and VanderWerff*, 1037
- See *Hitchings, Elion, VanderWerff, and Falco*, 765
- Elvehjem, C. A. See *Hankes, Henderson, Brickson, and Elvehjem* 873
- See *Olson, Fager, Burris, and Elvehjem*, 319
- Engel, Frank L., Pentz, E. Irene, and Engel, Mildred G. On the use of the nephrectomized rat for the study of rapid changes in nitrogen metabolism, 99

- Engel, Mildred G.** See *Engel, Pentz, and Engel*, 99  
**Entenman, C.** See *Chaikoff, Lerner, Entenman, and Dauben*, 1045

## F

- Fager, E. E. C.** See *Olson, Fager, Burris, and Elvehjem*, 319  
**Falco, Elvira A.** See *Hitchings, Elion, VanderWerff, and Falco*, 765  
**Feld, Emily A.** See *Nachmansohn, Rothenberg, and Feld*, 247  
**Ferger, Martha F., and du Vigneaud, Vincent.** The microbial growth inhibition produced by optical isomers of  $\beta$ -2-thienylalanine, 241  
**Fling, Marguerite.** See *Kobayashi, Fling, and Fox*, 391  
**Floyd, Curtis S.** See *Gjessing, Ludewig, and Chanutin*, 683  
**Flynn, Edwin H.** See *Carter, Belinsky, Clark, Flynn, Lytle, McCasland, and Robbins*, 415  
**Folch, Jordi.** The chemical structure of phosphatidyl serine, 439  
**Foote, Murray W.** See *Little, Sproston, and Foote*, 335  
**Fox, Sidney W.** See *Kobayashi, Fling, and Fox*, 391  
**Fraenkel-Conrat, Heinz, and Olcott, Harold S.** Reaction of formaldehyde with proteins. VI. Cross-linking of amino groups with phenol, imidazole, or indole groups, 827  
**Frantz, Ivan D., Jr., Zamecnik, Paul C., Reese, J. W., and Stephenson, Mary L.** The effect of dinitrophenol on the incorporation of alanine labeled with radioactive carbon into the proteins of slices of normal and malignant rat liver, 773  
**Freer, Richard M.** See *Dounce, Rothstein, Beyer, Meier, and Freer*, 361  
**Fried, Josef.** See *Titus and Fried*, 57  
**Friedgood, Harry B., Garst, Josephine B., and Haagen-Smit, A. J.** A new method for the separation of androgens from estrogens and for the partition of estriol from the estrone-estradiol fraction. With special reference to the identification and quantitative micro-determination of estrogens by ultraviolet absorption spectrophotometry, 523  
**Friedkin, Morris, and Lehninger, Albert L.** Phosphorylation coupled to electron transport between dihydroadipophosphopyridine nucleotide and oxygen, 757  
**Fruton, Joseph S.** See *Gulmann and Fruton*, 851  
 —. See *Simmonds and Fruton*, 705  
**Furst, Sidney S.** See *Brown, Petermann, and Furst*, 1043

## G

- Gallant, Dorothy Leaf.** See *Toennies and Gallant*, 451  
**Garber, M. J.** See *Schweigert, German, and Garber*, 383  
**Garst, Josephine B.** See *Friedgood, Garst, and Haagen-Smit*, 523  
**Genest, R. N.** See *Saunders, Siu, and Genest*, 697  
**German, H. L.** See *Schweigert, German, and Garber*, 383  
**Gjessing, Erland C., Ludewig, Stephan, and Chanutin, Alfred.** Fractionation, electrophoresis, and chemical studies of proteins in sera of control and injured goats, 683  
**Goodwin, Mari P.** See *Haines, Johnson, Goodwin, and Kuizenga*, 925  
**Goranson, E. S., Hamilton, J. E., and Haist, R. E.** Changes in phosphate and carbohydrate metabolism in shock, 1  
**Grant, W. Morton.** Observations on blood acetol after administration of acetol, methyl alcohol, and formaldehyde to rabbits, 93  
**Green, Arda Alden.** See *Rapport, Green, and Page*, 735  
**Greenberg, Leon A.** See *Lester and Greenberg*, 903  
**Greep, Roy O.** See *Perlmutter and Greep*, 915  
**Grinstein, Moisés, Kamen, Martin D., and Moore, Carl V.** Observation on the utilization of glycine in the biosynthesis of hemoglobin, 767

- Grisolia, Santiago.** See *Cohen and Grisolia*, 389  
 —. See *Mehler, Kornberg, Grisolia, and Ochoa*, 961  
**Groschke, A. C.** See *Bird, Rubin, and Groschke*, 611  
**Gutmann, Helmut R., and Fruton, Joseph S.** On the proteolytic enzymes of animal tissues. VIII. An intracellular enzyme related to chymotrypsin, 851
- H**
- Haagen-Smit, A. J.** See *Borsook, Deasy, Haagen-Smit, Keighley, and Lowy*, 1041  
 —. See *Friedgood, Garst, and Haagen-Smit*, 523  
**Haines, William J., Johnson, Richard H., Goodwin, Mari P., and Kuizenga, Marvin H.** Biochemical studies on hog testicular extract. I. Isolation and identification of 5-pregnen-3( $\beta$ )-ol-20-one, 925  
**Haist, R. E.** See *Goranson, Hamilton, and Haist*, 1  
**Halpern, George R., and Biely, Jacob.** The utilization of vitamin A in various carriers, 817  
**Hamilton, J. E.** See *Goranson, Hamilton, and Haist*, 1  
**Hankes, L. V., Henderson, L. M., Brickson, W. L., and Elvehjem, C. A.** Effect of amino acids on the growth of rats on niacin-tryptophan-deficient rations, 873  
**Harding, W. M., and Shive, William.** Biochemical transformations as determined by competitive analogue-metabolite growth inhibitions. VIII. An interrelationship of methionine and leucine, 743  
**Hauge, S. M.** See *Novak and Hauge*, 235, 647  
**Hegsted, D. Mark, and Lipmann, Fritz.** The pantothenic acid content of coenzyme A by chick assay, 89  
 —. See *Ling, Hegsted, and Stare*, 803  
**Henderson, L. M.** See *Hankes, Henderson, Brickson, and Elvehjem*, 873  
**Heuser, G. F.** See *Daniel, Scott, Heuser, and Norris*, 71  
**Hitchings, George H., Elion, Gertrude B., and VanderWerff, Henry.** The limitations of inhibition analysis, 1037  
 —, —, —, and **Falco, Elvira A.** Pyrimidine derivatives as antagonists of pteroylglutamic acid, 765  
**Hoberman, Henry D.** See *White, Hoberman, and Szego*, 1049  
**Holzman, George, and Niemann, Carl.** A spectrophotometric study of blood group A-specific substance isolated from hog gastric mucosa, 305  
**Horowitz, N. H., and Srb, Adrian M.** Growth inhibition of *Neurospora* by canavanine and its reversal, 371  
**Houlahan, Mary B.** See *Mitchell and Houlahan*, 883  
**Huebner, Charles F., and Jacobs, Walter A.** The aconite alkaloids. XXI. Further oxidation studies with atisine and isoatisine, 1001
- I**
- Irvin, Elinor Moore.** See *Irvin and Irvin*, 577, 589  
**Irvin, J. Logan, and Irvin Elinor Moore.** Apparent dissociation exponents of quinine, pamaquine, and a quinolylperidylcarbinol; application of an extended pH scale, 577  
 — and —. A fluorometric method for the determination of pamaquine, SN-13276, and SN-3294, 589  
**Jacobs, Walter A.** See *Huebner and Jacobs*, 1001  
**Jensen, Robert, Snellman, Olle, and Sylven, Bengt.** On the inhomogeneity of commercial heparin preparations from the physicochemical point of view, 265  
**Johnson, Marvin J.** See *Peterson and Johnson*, 775  
**Johnson, Richard H.** See *Haines, Johnson, Goodwin, and Kuizenga*, 925  
**Jones, Hardin B.** See *Reid and Jones*, 427

**Junqueira, Plinio B., and Schweigert, B. S.** Further studies on the rôle of vitamin B<sub>6</sub> in the metabolism of tryptophan by the rat, 605

K

**Kalckar, Herman M., Kjeldgaard, Niels Ole, and Klenow, Hans.** Inhibition of xanthine oxidase and related enzymes by 6-pteridyl aldehyde, 771

**Kamen, Martin D.** See *Grinstein, Kamen, and Moore*, 767

**Kaplan, Nathan O., and Lipmann, Fritz.** The assay and distribution of coenzyme A, 37

**Keighley, Geoffrey.** See *Borsook, Deasy, Haagen-Smit, Keighley, and Lowy*, 1041

**Kibrick, Andre C.** A polariscopic study of glutamic acid and pyrrolidonecarboxylic acid, 845

**Kielley, W. Wayne, and Meyerhof, Otto.** A new magnesium-activated adenosine-triphosphatase from muscle, 387

**King, Tsao E., and Cheldelin, Vernon H.** Pantothenic acid studies. IV. Propionic acid and  $\beta$ -alanine utilization, 273

**Kjeldgaard, Niels Ole.** See *Kalckar, Kjeldgaard, and Klenow*, 771

**Klenow, Hans.** See *Kalckar, Kjeldgaard, and Klenow*, 771

**Klotz, Irving M.** See *Melchior, Mellody, and Klotz*, 81

**Kobayashi, Yutaka, Fling, Marguerite, and Fox, Sidney W.** Antipodal specificity in the inhibition of growth of *Escherichia coli* by amino acids, 391

**Kornberg, Arthur.** Nucleotide pyrophosphatase and triphosphopyridine nucleotide structure, 1051

—, **Ochoa, Severo, and Mehler, Alan H.** Spectrophotometric studies on the decarboxylation of  $\beta$ -ketoacids, 159

—, See *Mehler, Kornberg, Grisolia, and Ochoa*, 961

—, See *Ochoa, Mehler, and Kornberg*, 979

**Krahl, M. E., and Park, C. R.** The uptake of glucose by the isolated diaphragm of normal and hypophysectomized rats, 939

**Kuizenga, Marvin H.** See *Haines, Johnson, Goodwin, and Kuizenga*, 925

**Kun, Ernest.** Inhibition of the phosphorylation of glucose by meningococcal endotoxin, 761

L

**Lauffer, Max A.** Interaction between tobacco mosaic virus and bovine serum albumin, 481

**Lehninger, Albert L.** See *Friedkin and Lehninger*, 757

**Lepow, Jean Papell.** See *Sealock and Lepow*, 763

**Lerner, S. R.** See *Chaikoff, Lerner, Entenman, and Dauben*, 1045

**Lester, David, and Greenberg, Leon A.** The determination of acetone and ketone bodies, 903

**Ling, Chiun-Tong, Hegsted, D. Mark, and Stare, F. J.** The effect of pyridoxine deficiency on the tryptophan-niacin transformation in rats, 803

**Ling, Kuo-Huang, and Tung, Ta-Cheng.** The oxidative demethylation of monomethyl-L-amino acids, 643

**Lipmann, Fritz.** See *Hegsted and Lipmann*, 89

—, See *Kaplan and Lipmann*, 37

**Little, John E., Sproston, Thomas J., and Foote, Murray W.** Isolation and antifungal action of naturally occurring 2-methoxy-1,4-naphthoquinone, 335

**Logan, Milan A.** See *Boyd, Logan, and Tytell*, 1013, 1027

**Loo, Y. H.** See *Carter and Loo*, 723

**Loring, Hubert S., Roll, Paul M., and Pierce, John G.** A simplified procedure for the preparation of cytidylic acid and diammonium uridyate from yeast nucleic acid, 729

**Lowry, Oliver H.** See *Burch, Lowry, Bessey, and Berson*, 791

- Lowy, Peter H.** See *Borsook, Deasy, Haagen-Smit, Keighley, and Lowy*, 1041
- Luck, J. Murray.** See *Teresi and Luck*, 653
- Ludewig, Stephan.** See *Gjessing, Ludewig, and Chanutin*, 683
- Lytle, Betty.** See *Carter, Belinsky, Clark, Flynn, Lytle, McCasland, and Robbins*, 415

## M

- Magasanik, Boris, and Chargaff, Erwin.** The stereochemistry of an enzymatic reaction: the oxidation of *l*-, *d*-, and *epi*-inositol by *Acetobacter suboxydans*, 173
- Martin, Gustav J., Avakian, Souren, and Moss, Jack.** Studies of pyridoxine displacement, 495  
— See *Beiler and Martin*, 31
- Maw, George A., and du Vigneaud, Vincent.** Dimethyl- $\beta$ -propiothetin, a new methyl donor, 381
- McCasland, G. E.** See *Carter, Belinsky, Clark, Flynn, Lytle, McCasland, and Robbins*, 415
- McGilvery, R. W., and Cohen, Philip P.** The decarboxylation of *L*-phenylalanine by *Streptococcus faecalis* R, 813
- Mehler, Alan H., Kornberg, Arthur, Grisolia, Santiago, and Ochoa, Severo.** The enzymatic mechanism of oxidation-reductions between malate or isocitrate and pyruvate, 961  
— See *Kornberg, Ochoa, and Mehler*, 159  
— See *Ochoa, Mehler, and Kornberg*, 979
- Meier, Rebecca.** See *Dounce, Rothstein, Beyer, Meier, and Freer*, 361
- Melchior, Jacklyn B., Melody, Margaret, and Klotz, Irving M.** The synthesis of protein by non-proliferating *Escherichia coli*, 81
- Melody, Margaret.** See *Melchior, Melody, and Klotz*, 81
- Meyerhof, Otto.** See *Kielley and Meyerhof*, 387
- Mills, Mary B.** See *Roe, Mills, Oesterling, and Damron*, 201

- Minovitch, Eve.** See *Sinclair*, 343, 355
- Mitchell, Herschel K., and Houlahan, Mary B.** An intermediate in the biosynthesis of lysine in *Neurospora*, 883
- Moore, Carl V.** See *Grinstein, Kamen, and Moore*, 767
- Moss, Jack.** See *Martin, Avakian, and Moss*, 495
- Moyer, A. W.** See *du Vigneaud, Moyer, and Chandler*, 477
- Mullins, Lorin J., and Zerahn, Karl.** The distribution of potassium isotopes in biological material, 107

## N

- Nachmansohn, David, Rothenberg, Mortimer A., and Feld, Emily A.** Studies on cholinesterase. V. Kinetics of the enzyme inhibition, 247
- Naganna, B., and Narayana menon, V. K.** Erythrocyte pyrophosphatase in health and disease. I. Properties of the enzyme, 501
- Narayana menon, V. K.** See *Naganna and Narayana menon*, 501
- Niemann, Carl.** See *Holzman and Niemann*, 305
- Norris, L. C.** See *Daniel, Scott, Heuser, and Norris*, 71
- Novak, A. F., and Hauge, S. M.** Isolation of the unidentified growth factor (vitamin B<sub>12</sub>) in distillers' dried solubles, 647  
— and —. Some properties of an unidentified growth factor in distillers' dried solubles, 235

## O

- Ochoa, Severo.** Biosynthesis of tricarboxylic acids by carbon dioxide fixation. I. The preparation and properties of oxalosuccinic acid, 115  
— III. Enzymatic mechanisms, 133  
—, **Mehler, Alan H., and Kornberg, Arthur.** Biosynthesis of dicarboxylic acids by carbon dioxide fixation. I.

- Isolation and properties of an enzyme from pigeon liver catalyzing the reversible oxidative decarboxylation of l-malic acid, 979
- and Weisz-Tabori, Erna. Biosynthesis of tricarboxylic acids by carbon dioxide fixation. II. Oxalosuccinic carboxylase, 123
- See Kornberg, Ochoa, and Mehler, 159
- See Mehler, Kornberg, Grisolia, and Ochoa, 961
- Oesterling, M. Jane. See Roe, Mills, Oesterling, and Damron, 201
- Olcott, Harold S. See Fraenkel-Conrat and Olcott, 827
- Olson, O. E., Fager, E. E. C., Burris, R. H., and Elvehjem, C. A. Folic acid activity in homogenates of rat liver, 319
- Ott, Melvin L. See Dorfman, Ott, and Whitney, 621
- Ott, Walther H., Rickes, Edward L., and Wood, Thomas R. Activity of crystalline vitamin B<sub>12</sub> for chick growth, 1047
- Overman, Ralph S., and Wright, Irving S. A new blood clotting inhibitor, 759

## P

- Page, Irvine H. See Rapport, Green, and Page, 735
- Park, C. R. See Krahl and Park, 939
- Pentz, E. Irene. See Engel, Pentz, and Engel, 99
- Perlmutter, Martin, and Greep, Roy O. The uptake of glucose and the synthesis of glycogen by the isolated diaphragm of normal and pituitectomized rats, 915
- Petermann, Mary L. See Brown, Petermann, and Furst, 1043
- Peterson, Merlin H., and Johnson, Marvin J. The estimation of fatty acids of intermediate chain length by partition chromatography, 775
- Pierce, John G. See Loring, Roll, and Pierce, 729
- Pilhorn, Harry R. See Bischoff and Pilhorn, 663
- Pillemer, Louis. See Wittler and Pillemer, 23
- Pringle, A. See Woolley and Pringle, 327

## R

- Rapport, Maurice M., Green, Arda Alden, and Page, Irvine H. Partial purification of the vasoconstrictor in beef serum, 735
- Rease, J. W. See Frantz, Zamecnik, Reese, and Stephenson, 773
- Reid, James C., and Jones, Hardin B. Radioactivity distribution in the tissues of mice bearing melanosarcoma after administration of DL-tyrosine labeled with radioactive carbon, 427
- Rickes, Edward L. See Ott, Rickes, and Wood, 1047
- Robbins, Mary. See Carter, Belinsky, Clark, Flynn, Lytle, McCasland, and Robbins, 415
- Roberts, Sidney, Adams, Elijah, and White, Abraham. Release of antibody by lymphoid tissue *in vitro*, 379
- Roe, Joseph H., Mills, Mary B., Oesterling, M. Jane, and Damron, Charlotte M. The determination of diketo-l-gulonic acid, dehydro-l-ascorbic acid, and l-ascorbic acid in the same tissue extract by the 2,4-dinitrophenylhydrazine method, 201
- Roll, Paul M. See Loring, Roll, and Pierce, 729
- Rothenberg, Mortimer A. See Nachmansohn, Rothenberg, and Feld, 247
- Rothstein, Aser. See Dounce, Rothstein, Beyer, Meier, and Freer, 361
- Rubin, Max. See Bird, Rubin, and Groschke, 611

## S

- Saltzman, Abraham. Fluorophotometric method for the estimation of salicylate in blood, 399



- Sato, Yoshio, Barry, Guy T., and Craig, Lyman C.** Distribution studies. XI. Isolation of benzylpenicillin containing radioactive sulfur, 217  
— See *Barry, Sato, and Craig*, 209, 221
- Saunders, P. R., Siu, R. G. H., and Genest, R. N.** A cellulolytic enzyme preparation from *Myrothecium verrucaria*, 697
- Schenker, Florence.** See *Daniel, Scott, Heuser, and Norris*, 71
- Schweigert, B. S., German, H. L., and Garber, M. J.** Synthesis of nicotinic acid from tryptophan by the developing chick embryo, 383  
— See *Junqueira and Schweigert*, 605
- Schwert, George W.** A note on the application of the colorimetric ninhydrin determination to studies of enzyme kinetics, 411
- Scott, M. L.** See *Daniel, Scott, Heuser, and Norris*, 71
- Sealock, Robert Ridgely, and Lepow, Jean Papell.** Antipernicious anemia extracts and tyrosine metabolism in the scorbutic guinea pig, 763
- Seegers, Walter H.** See *Ware and Seegers*, 565
- Shaffer, C. Boyd, and Critchfield, Frances H.** Lipotropic activity and toxicity of methoxinine (oxymethionine), 489
- Shive, William.** See *Harding and Shive*, 743
- Simmonds, Sofia.** Utilization of sulfur-containing amino acids by mutant strains of *Escherichia coli*, 717  
— and *Fruton, Joseph S.* The utilization of proline derivatives by mutant strains of *Escherichia coli*, 705
- Sinclair, R. G.** The lecithin, cephalin, and sphingomyelin content of serum. I. As indicated by the choline-phosphorus and nitrogen-phosphorus ratios, 343  
— II. As indicated by the amino nitrogen and non-diffusible phosphorus content after differential hydrolysis of the acetone-insoluble lipides, 355
- Singer, Leon.** See *Comar, Davis, and Singer*, 905
- Singer, Thomas P.** On the mechanism of enzyme inhibition by sulfhydryl reagents, 11
- Siu, R. G. H.** See *Saunders, Siu, and Genest*, 697
- Snell, Esmond E.** See *Volcani and Snell*, 893
- Snellman, Olle.** See *Jensen, Snellman, and Sylvén*, 265
- Somogyi, Michael.** Studies of arteriovenous differences in blood sugar. I. Effect of alimentary hyperglycemia on the rate of extrahepatic glucose assimilation, 189  
— II. Effect of hypoglycemia on the rate of extrahepatic glucose assimilation, 597
- Sproston, Thomas J.** See *Little, Sproston, and Foote*, 335
- Srb, Adrian M.** See *Horowitz and Srb*, 371
- Stadtman, E. R., and Barker, H. A.** Two new reactions producing acetyl phosphate, 1039
- Stare, F. J.** See *Ling, Hegsted, and Stare*, 803
- Stephenson, Mary L.** See *Frantz, Zamecnik, Reese, and Stephenson*, 773
- Strachman, Norma.** See *Bodansky*, 465
- Sylvén, Bengt.** See *Jensen, Snellman, and Sylvén*, 265
- Szego, Clara M.** See *White, Hoberman, and Szego*, 1049

## T

- Teresi, J. D., and Luck, J. Murray.** The combination of organic anions with serum albumin. VI. Quantitative studies by equilibrium dialysis, 653
- Titus, Elwood, and Fried, Josef.** Counter-current distribution studies on streptomycin: the tautomerism of streptomycin, 57
- Toennies, Gerrit, and Gallant, Dorothy Leaf.** Bacteriometric studies. I. Factors affecting the precision of bacterial

- growth responses and their measurement, 451  
 —. See *Winegard and Toennies*, 45  
*Tsuyuki, Henry*. See *Sinclair*, 343, 355  
*Tung, Ta-Cheng*. See *Ling and Tung*, 643  
*Tytell, Alfred A.* See *Boyd, Logan, and Tytell*, 1013, 1027

## V

- VanderWerff, Henry*. See *Hitchings, Elion, and VanderWerff*, 1037  
 —. See *Hitchings, Elion, VanderWerff, and Falco*, 765  
*du Vigneaud, Vincent, Moyer, A. W., and Chandler, Joseph P.* Dimethylthetin as a biological methyl donor, 477  
 —. See *Ferger and du Vigneaud*, 241  
 —. See *Maw and du Vigneaud*, 381  
*Volcani, Benjamin E., and Snell, Esmond E.* The effects of canavanine, arginine, and related compounds on the growth of bacteria, 893

## W

- Ware, Arnold G., and Seegers, Walter H.* Studies on prothrombin: purification, inactivation with thrombin, and activation with thromboplastin and calcium, 565  
*Weisz-Tabori, Erna*. See *Ochoa and Weisz-Tabori*, 123  
*White, Abraham, Hoberman, Henry D., and Szego, Clara M.* Influence of adrenalectomy and fasting on the incorporation of isotopic nitrogen into the tissues of mice, 1049  
 —. See *Roberts, Adams, and White*, 379

- Whiteley, Arthur H.* A microvolumetric Van Slyke blood gas apparatus, 947  
*Whitney, Richard*. See *Dorfman, Ott, and Whitney*, 621  
*Wilbur, Karl M.* See *Bernheim, Bernheim, and Wilbur*, 257  
*Williamson, Martin B.* The vitamin A content of fetal rats from mothers on a high cholesterol diet, 631  
 — and *Appel, Muriel*. The effect of sulfonamide in the diet of pregnant rats on erythropoiesis in the fetuses, 769  
*Winegard, Herbert M., and Toennies, Gerrit*. A method for computing the effective extraneous reducers in the Folin spectrophotometric determination of cystine, 45  
*Wittler, Ruth G., and Pillemer, Louis*. The immunochemistry of toxins and toxoids. V. The solubility of staphylococcal toxin in methanol-water mixtures under controlled conditions of pH, ionic strength, and temperature, 23  
*Wolff, J., and Chaikoff, I. L.* Plasma inorganic iodide as a homeostatic regulator of thyroid function, 555  
*Wood, Thomas R.* See *Ott, Rickes, and Wood*, 1047  
*Woolley, D. W., and Pringle, A.* Some structural analogues antagonistic to pteroylglutamic acid, 327  
*Wright, Irving S.* See *Overman and Wright*, 759

## Z

- Zamecnik, Paul C.* See *Frantz, Zamecnik, Reese, and Stephenson*, 773  
*Zerahn, Karl*. See *Mullins and Zerahn*, 107



# INDEX TO SUBJECTS

## A

- Acetobacter suboxydans:** Inositol isomers oxidation, rôle, *Magasanik and Chargaff*, 173  
— oxidation, *Carter, Belinsky, Clark, Flynn, Lytle, McCasland, and Robbins*, 415
- Acetol:** Blood, acetol, methyl alcohol, and formaldehyde administration, effect, *Grant*, 93
- Acetone:** Determination, *Lester and Greenberg*, 903
- Acetyl phosphate:** Production, *Stadtman and Barker*, 1039
- Aconite:** Alkaloids, *Huebner and Jacobs*, 1001
- Adenine:** Desoxypentose nucleic acid, incorporation, *Brown, Petermann, and Furst*, 1043  
Pentose nucleic acid, incorporation, *Brown, Petermann, and Furst*, 1043
- Adenosinetriphosphatase:** Muscle, magnesium-activated, *Kielley and Meyerhof*, 387
- Adenosine triphosphate:** Purified, preparation, *Dounce, Rothstein, Beyer, Meier, and Freer*, 361
- Adrenalectomy:** Tissue nitrogen, isotopic, effect, *White, Hoberman, and Szego*, 1049
- Alanine:**  $\beta$ -, utilization, pantothenic acid relation, *King and Cheldelin*, 273  
DL-, phosphatase, intestine, bone, and kidney, effect, *Bodansky*, 465  
Phenyl-. See Phenylalanine  
Radioactive carbon-labeled, liver protein, incorporation, dinitrophenol effect, *Frantz, Zamecnik, Reese, and Stephenson*, 773  
 $\beta$ -2-Thienyl-, optical isomers, microbial growth, effect, *Ferger and du Vigneaud*, 241
- Albumin:** Blood serum, organic anions, combination, *Teresi and Luck*, 653  
— — —, combination, equilibrium dialysis, *Teresi and Luck*, 653  
— — —, tobacco mosaic virus and, interaction, *Lauffer*, 481
- Alcohol:** Methyl. See Methyl alcohol
- Alkaloid(s):** Aconite, *Huebner and Jacobs*, 1001
- Alloxan:** Diabetes, palmitic acid labeled with radioactive carbon, glucose from, *Chaikoff, Lerner, Entenman, and Dauben*, 1045
- Amino acid(s):** Determination, *Clostridium perfringens* use, *Boyd, Logan, and Tytell*, 1027  
*Escherichia coli* growth, specificity, *Kobayashi, Fling, and Fox*, 391  
Monomethyl-L-, demethylation, oxidative, *Ling and Tung*, 643  
Niacin-tryptophan-deficient diets, growth, effect, *Hankes, Henderson, Brickson, and Elvehjem*, 873  
Sulfur-containing, *Escherichia coli* mutant strains, utilization, *Simmonds*, 717
- Amino group(s):** Phenol, imidazole, or indole groups, cross-linking, *Fraenkel-Conrat and Olcott*, 827
- Androgen(s):** Estrogens, separation, *Friedgood, Garst, and Haagen-Smit*, 523
- Anemia:** Antipernicious, extracts, tyrosine metabolism and, scurvy, relation, *Sealock and Lepow*, 763
- Antibody:** Lymphoid tissue, release, *Roberts, Adams, and White*, 379
- Apparatus:** Van Slyke, blood gas, microvolumetric, *Whiteley*, 947
- Arginase:** Cell nucleus, *Dounce and Beyer*, 859
- Arginine:** Bacterial growth, effect, *Volcani and Snell*, 893
- Ascorbic acid:** Dehydro-l-, determination, 2,4-dinitrophenylhydrazine method, *Roe, Mills, Oesterling, and Damron*, 201  
l-, determination, 2,4-dinitrophenylhydrazine method, *Roe, Mills, Oesterling, and Damron*, 201
- Atisine:** Iso-, oxidation, *Huebner and Jacobs*, 1001  
Oxidation, *Huebner and Jacobs*, 1001

- A**
- B**
- Bacillus:** See also *Lactobacillus*
- Bacteria:** Growth, canavanine and arginine, effect, *Volcani and Snell*, 893  
—, measurement, factors affecting, *Toennies and Gallant*, 451  
Viruses, synthesis, *Cohen*, 281, 295  
See also *Acetobacter*, *Clostridium*, *Escherichia*, *Meningococcus*, *Staphylococcus*, *Streptococcus*
- Bacterimetric studies:** *Toennies and Gallant*, 451
- Bacteriophage(s):** Desoxyribonucleic acid, phosphorus origin, *Cohen*, 295  
*Escherichia coli* infected with, nucleic acid and protein synthesis, *Cohen*, 281
- Barbituric acid:** Thio-, lipide oxidation products and, reaction, *Bernheim, Bernheim, and Wilbur*, 257
- Benzylpenicillin:** Radioactive sulfur-containing, isolation, counter-current distribution use, *Sato, Barry, and Craig*, 217
- Blood:** Acetol, acetol, methyl alcohol, and formaldehyde administration, effect, *Grant*, 93  
Coagulation, inhibitor, *Overman and Wright*, 759  
Gas, Van Slyke microvolumetric apparatus, *Whiteley*, 947  
Group A-specific substance, gastric mucosa, *Holzman and Niemann*, 305  
Hyaluronidase inhibitor, *Dorfman, Ott, and Whitney*, 621  
Salicylate, determination, fluorophotometric, *Saltzman*, 399
- Blood cell:** Red, pyrophosphatase, *Naganna and Narayana menon*, 501
- Blood plasma:** Iodide, inorganic, thyroid function, relation, *Wolff and Chaikoff*, 555
- Blood serum:** Albumin, organic anions, combination, *Terest and Luck*, 653  
—, —, —, equilibrium dialysis, *Terest and Luck*, 653  
—, tobacco mosaic virus and, interaction, *Lauffer*, 481

- Blood serum—continued:**
- Cephalin, *Sinclair*, 343, 355  
 $\alpha$ -Estradiol solubility, *Bischoff and Pilhorn*, 663  
Iron, determination, micro-, *Burch, Lowry, Bessey, and Berson*, 791  
Lecithin, *Sinclair*, 343, 355  
Progesterone solubility, *Bischoff and Pilhorn*, 663  
Proteins, injury effect, *Gjessing, Ludewig, and Chanutin*, 683  
Sphingomyelin, *Sinclair*, 343, 355  
Testosterone solubility, *Bischoff and Pilhorn*, 663  
Vasoconstrictor, purification, *Rapport, Green, and Page*, 735
- Blood sugar:** Arteriovenous differences, *Somogyi*, 189, 597  
See also Hyperglycemia, Hypoglycemia
- Bone:** Phosphatase, DL-alanine, L-glutamic acid, L-lysine, and L-histidine, effect, *Bodansky*, 465

## C

- Calcium:** Prothrombin, effect, *Ware and Seegers*, 565
- Canavanine:** Bacterial growth, effect, *Volcani and Snell*, 893  
*Neurospora* growth, effect, *Horowitz and Srb*, 371
- Carbamyl-L-glutamic acid:** Citrulline synthesis, rôle, *Cohen and Grisolia*, 389
- Carbinol:** Quinolylpiperidyl-, dissociation exponents, *Irvin and Irvin*, 577
- Carbohydrate(s):** Metabolism, shock effect, *Goranson, Hamilton, and Haist*, 1
- Carbon:** Radioactive, alanine labeled with, liver protein, incorporation, dinitrophenol effect, *Frantz, Zamecnik, Reese, and Stephenson*, 773  
—, palmitic acid labeled with, glucose from, alloxan diabetes, *Chaikoff, Lerner, Entenman, and Dauben*, 1045  
—, DL-tyrosine labeled with, melanoma, distribution, *Reid and Jones*, 427

**Carbon dioxide:** Fixation, dicarboxylic acid biosynthesis, rôle, *Ochoa, Mehler, and Kornberg*, 979  
 —, tricarboxylic acid synthesis, rôle, *Ochoa*, 115  
*Ochoa and Weisz-Tabori*, 123  
*Ochoa*, 133  
 —, — acids, enzymatic mechanisms, *Ochoa*, 133  
**Carboxylase:** Oxalosuccinic, *Ochoa and Weisz-Tabori*, 123  
**Carboxylic acid(s):** Di-, biosynthesis, carbon dioxide fixation rôle, *Ochoa, Mehler, and Kornberg*, 979  
 Pyrrolidone-, polarimetry, *Kibrick*, 845  
 Tri-, biosynthesis, carbon dioxide fixation rôle, *Ochoa*, 115  
*Ochoa and Weisz-Tabori*, 123  
*Ochoa*, 133  
 —, carbon dioxide fixation, enzymatic mechanisms, *Ochoa*, 133  
**Cell:** Nucleus, arginase, *Dounce and Beyer*, 859  
**Cephalin:** Blood serum, *Sinclair*, 343  
**Chick:** Growth factor, manure, *Bird, Rubin, and Groschke*, 611  
 — —, stability and solubility, *Bird, Rubin, and Groschke*, 611  
 —, vitamin B<sub>12</sub>, crystalline, effect, *Ott, Rickes, and Wood*, 1047  
**Cholesterol:** -High maternal diet, fetus vitamin A, effect, *Williamson*, 631  
**Cholinesterase:** *Nachmansohn, Rothenberg, and Feld*, 247  
 Inhibition, kinetics, *Nachmansohn, Rothenberg, and Feld*, 247  
**Chymotrypsin:** Enzyme, intracellular, relation, *Gutmann and Fruton*, 851  
**Citrulline:** Synthesis, carbamyl-L-glutamic acid rôle, *Cohen and Grisolia*, 389  
**Clostridium perfringens:** Amino acid determination, use, *Boyd, Logan, and Tytell*, 1027  
 Growth requirements, *Boyd, Logan, and Tytell*, 1013  
**Coagulation:** Blood, inhibitor, *Overman and Wright*, 759

**Coenzyme:** A, determination and distribution, *Kaplan and Lipmann*, 37  
 —, pantothenic acid, determination, *Hegsted and Lipmann*, 89  
**Copper:** Radioactive, fate, cattle, *Comar, Davis, and Singer*, 905  
**Cystine:** Determination, spectrophotometric, Folin, reducers, computation, *Winegard and Toennies*, 45  
**Cytidylic acid:** Nucleic acid, yeast, preparation, *Loring, Roll, and Pierce*, 729

D

**Dehydro-l-ascorbic acid:** Determination, 2,4-dinitrophenylhydrazine method, *Roe, Mills, Oesterling, and Damron*, 201  
**Desoxyribose nucleic acid:** Adenine incorporation, *Brown, Petermann, and Furst*, 1043  
**Desoxyribonucleic acid(s):** Bacteriophages, phosphorus origin, *Cohen*, 295  
**Diabetes:** Alloxan, palmitic acid labeled with radioactive carbon, glucose from, *Chaikoff, Lerner, Entenman, and Dauben*, 1045  
**Diammonium uridylate:** Nucleic acid, yeast, preparation, *Loring, Roll, and Pierce*, 729  
**Diaphragm:** Glycogen synthesis, hypophysectomy effect, *Perlmutter and Greep*, 915  
 — uptake, hypophysectomy effect, *Perlmutter and Greep*, 915  
*Krahl and Park*, 939  
**Dicarboxylic acid(s):** Biosynthesis, carbon dioxide fixation rôle, *Ochoa, Mehler, and Kornberg*, 979  
**Dihydrodiphosphopyridine:** Nucleotide, oxygen and, electron transport, coupled phosphorylation, *Friedkin and Lehninger*, 757  
**Diketo-l-gulonic acid:** Determination, 2,4-dinitrophenylhydrazine method, *Roe, Mills, Oesterling, and Damron*, 201  
**Dimethyl- $\beta$ -propiolactone:** Methyl donor, *Maw and du Vigneaud*, 381

- Dimethylthetin:** Methyl donor, *du Vigneaud, Moyer, and Chandler*, 477
- Dinitrophenol:** Liver protein, radio-active carbon-labeled alanine incorporation, effect, *Frantz, Zamecnik, Reese, and Stephenson*, 773
- Dinitrophenylhydrazine:** 2,4-, diketo-l-gulonic acid, dehydro-l-ascorbic acid, and l-ascorbic acid determination, use in, *Roe, Mills, Oesterling, and Damron*, 201
- Distillers' soluble(s):** Growth factor, *Novak and Hauge*, 235  
— —, isolation, *Novak and Hauge*, 647
- Distribution studies:** *Barry, Sato, and Craig*, 209  
*Sato, Barry, and Craig*, 217  
*Barry, Sato, and Craig*, 221

## E

- Embryo:** Chick, nicotinic acid synthesis, tryptophan relation, *Schweigert, German, and Garber*, 383
- Enzyme(s):** Carbon dioxide fixation, tricarboxylic acids, mechanisms, *Ochoa*, 133  
Inhibition, sulfhydryl reagents, mechanism, *Singer*, 11  
Isocitrate-pyruvate oxidation-reduction, mechanism, *Mehler, Kornberg, Grisolia, and Ochoa*, 961  
Kinetics, colorimetric ninhydrin determination, use in study, *Schwert*, 411  
Liver, l-malic acid decarboxylation, effect, *Ochoa, Mehler, and Kornberg*, 979  
Malate-pyruvate oxidation-reduction, mechanism, *Mehler, Kornberg, Grisolia, and Ochoa*, 961  
*Myrothecium verrucaria*, *Saunders, Siu, and Genest*, 697  
Proteolytic, tissue, *Gutmann and Fruton*, 851  
*See also* Adenosinetriphosphatase, Arginase, Carboxylase, Cholinesterase, Chymotrypsin, Hyaluronidase, Oxidase, Phosphatase, Pyrophosphatase
- Erythrocyte(s):** *See* Blood cell, red
- Erythropoiesis:** Fetus, sulfonamide in maternal diet, effect, *Williamson and Appel*, 769
- Escherichia coli:** B, bacteriophage-infected, nucleic acid and protein synthesis, *Cohen*, 281  
Growth, amino acid specificity, *Kobayashi, Fling, and Fox*, 391  
Mutant strains, proline derivatives, utilization, *Simmonds and Fruton*, 705  
— —, sulfur-containing amino acids, utilization, *Simmonds*, 717  
Protein synthesis, effect, *Melchior, Melody, and Klotz*, 81
- Esterase:** Cholin-. *See* Cholinesterase
- Estradiol:**  $\alpha$ -, blood serum solubility, *Bischoff and Pilhorn*, 663  
—, protein solution solubility, *Bischoff and Pilhorn*, 663  
—, salt solution solubility, *Bischoff and Pilhorn*, 663
- Estrone-**, fraction, estriol partition, *Friedgood, Garst, and Haagen-Smit*, 523
- Estriol:** Estrone-estradiol fraction, partition, *Friedgood, Garst, and Haagen-Smit*, 523
- Estrogen(s):** Androgens, separation, *Friedgood, Garst, and Haagen-Smit*, 523  
Determination, micro-, spectrophotometric, *Friedgood, Garst, and Haagen-Smit*, 523
- Estrone:** Estradiol fraction, estriol partition, *Friedgood, Garst, and Haagen-Smit*, 523

## F

- Fasting:** Tissue nitrogen, isotopic, effect, *White, Hoberman, and Szego*, 1049
- Fatty acid(s):** Determination, chromatographic, *Peterson and Johnson*, 775
- Fetus:** Erythropoiesis, sulfonamide in maternal diet, effect, *Williamson and Appel*, 769  
Vitamin A, cholesterol-high maternal diet, effect, *Williamson*, 631
- Folic acid:** Liver, *Olson, Fager, Burris, and Elvehjem*, 319

- Formaldehyde:** Blood acetol, administration effect, *Grant*, 93  
 -Protein reaction, *Fraenkel-Conrat and Olcott*, 827  
**Fungus:** 2-Methoxy-1,4-naphthoquinone action, *Little, Sproston, and Foote*, 335

*See also Myrothecium*

## G

- Gas:** Blood, Van Slyke microvolumetric apparatus, *Whiteley*, 947  
**Glucose:** Extrahepatic, assimilation, alimentary hyperglycemia, relation, *Somogyi*, 189  
 —, —, hypoglycemia, relation, *Somogyi*, 597  
 Palmitic acid conversion to, radioactive carbon-labeled, alloxan diabetes, *Chaikoff, Lerner, Entenman, and Dauben*, 1045  
 Phosphorylation, meningococcal endotoxin effect, *Kun*, 761  
 Uptake, diaphragm, hypophysectomy effect, *Perlmutter and Greep*, 915  
*Krahl and Park*, 930  
**Glutamic acid:** Carbamyl-L-, citrulline synthesis, rôle, *Cohen and Grisolia*, 389  
 L-, phosphatase, intestine, bone, and kidney, effect, *Bodansky*, 465  
 Polarimetry, *Kibrick*, 845  
 Pteroyl-. *See* Pteroylglutamic acid  
**Glycine:** Hemoglobin biosynthesis, use, *Grinstein, Kamen, and Moore*, 767  
**Glycogen:** Synthesis, diaphragm, hypophysectomy effect, *Perlmutter and Greep*, 915  
**Growth:** Bacteria, canavanine and arginine, effect, *Volcani and Snell*, 893  
 —, measurement, factors affecting, *Toennies and Gallant*, 451  
 Biochemical transformations, *Harding and Shive*, 743  
 Chick, vitamin B<sub>12</sub>, crystalline, effect, *Ott, Rickes, and Wood*, 1047  
*Clostridium perfringens*, *Boyd, Logan, and Tytell*, 1013  
*Escherichia coli*, amino acid specificity, *Kobayashi, Fling, and Fox*, 391  
 Factor, *Colio and Babb*, 405

## Growth—continued:

- Factor, chick, manure, *Bird, Rubin, and Groschke*, 611  
 —, —, —, stability and solubility, *Bird, Rubin, and Groschke*, 611  
 —, distillers' solubles, *Novak and Hauge*, 647  
 —, —, —, isolation, *Novak and Hauge*, 235  
 Methionine-deficient diet, DL-homoserine effect, *Armstrong and Binkley*, 889  
 Methionine-leucine relation, *Harding and Shive*, 743  
 Microbial,  $\beta$ -2-thienylalanine effect, *Ferger and du Vigneaud*, 241  
*Neurospora*, canavanine, effect, *Horowitz and Srb*, 371  
 Niacin - tryptophan - deficient diets, amino acids, effect, *Hankes, Henderson, Brickson, and Elvehjem*, 873  
 D-Tyrosine effect, *Bubl and Butts*, 637  
**Gulonic acid:** Diketo-L-, determination, 2,4-dinitrophenylhydrazine method, *Roe, Mills, Oesterling, and Damron*, 201

## H

- Hemoglobin:** Biosynthesis, glycine use, *Grinstein, Kamen, and Moore*, 767  
**Heparin:** Preparations, inhomogeneity, *Jensen, Snellman, and Sylven*, 265  
**Hesperidin:** Hyaluronidase, effect, *Beiler and Martin*, 31  
**Histidine:** L-, phosphatase, intestine, bone, and kidney, effect, *Bodansky*, 465  
**Homoserine:** DL-, methionine-deficient diet, growth, effect, *Armstrong and Binkley*, 889  
**Hormone(s):** Steroid, biological systems, *Bischoff and Pilhorn*, 663  
**Hyaluronidase:** Blood, inhibitor, *Dorfman, Ott, and Whitney*, 621  
 Hesperidin effect, *Beiler and Martin*, 31  
**Hydroxy-L-proline:** Oxidation, periodate effect, *Carter and Loo*, 723



- Hyperglycemia:** Alimentary, glucose, extrahepatic, assimilation, relation, *Somogyi*, 189
- Hypoglycemia:** Glucose, extrahepatic, assimilation, *Somogyi*, 597
- Hypophysectomy:** Diaphragm glucose uptake, effect, *Perlmutter and Greep*, 915  
*Krahl and Park*, 939  
 —, glycogen synthesis, effect, *Perlmutter and Greep*, 915
- I**
- Imidazole:** Amino groups, cross-linking, *Fraenkel-Conrat and Olcott*, 827
- Indole group(s):** Amino groups, cross-linking, *Fraenkel-Conrat and Olcott*, 827
- Inhibition:** Analysis, limitations, *Hitchings, Elion, and VanderWerff*, 1037
- Injury:** Blood serum proteins, effect, *Gjessing, Ludwig, and Chanutin*, 683
- Inositol:** Isomers, oxidation, *Acetobacter suboxydans* rôle, *Magasanik and Chargaff*, 173  
 Oxidation, *Acetobacter suboxydans*, *Carter, Belinsky, Clark, Flynn, Lytle, McCasland, and Robbins*, 415
- Intestine:** Phosphatase, DL-alanine, L-glutamic acid, L-lysine, and L-histidine, effect, *Bodansky*, 465
- Iodide:** Inorganic, blood plasma, thyroid function, relation, *Wolff and Chaikoff*, 555
- Iron:** Blood serum, determination, micro-, *Burch, Lowry, Bessey, and Berson*, 791
- Isoatisine:** Oxidation, *Huebner and Jacobs*, 1001
- Isocitrate:** -Pyruvate oxidation-reduction, enzymatic mechanism, *Mehler, Kornberg, Grisolia, and Ochoa*, 961
- K**
- Keto acid(s):**  $\beta$ -, decarboxylation, spectrophotometry, *Kornberg, Ochoa, and Mehler*, 159
- Ketone bodies:** Determination, *Lester and Greenberg*, 903
- Kidney:** Phosphatase, DL-alanine, L-glutamic acid, L-lysine, and L-histidine, effect, *Bodansky*, 465  
*See also Nephrectomy*
- L**
- Lactobacillus casei:** Nutrition, factors, *Daniel, Scott, Heuser, and Norris*, 71
- Lecithin:** Blood serum, *Sinclair*, 343
- Leucine:** Growth, methionine relation, *Harding and Shive*, 743  
 Liver homogenate peptide, relation, *Borsook, Deasy, Haagen-Smit, Keighley, and Lowy*, 1041
- Lipide(s):** Oxidation products, thiobarbituric acid and, reaction, *Bernheim, Bernheim, and Wilbur*, 257
- Liver:** Enzyme, L-malic acid decarboxylation, effect, *Ochoa, Mehler, and Kornberg*, 979  
 Folic acid, *Olson, Fager, Burris, and Elvehjem*, 319  
 Homogenate peptide, isolation, *Borsook, Deasy, Haagen-Smit, Keighley, and Lowy*, 1041  
 — —, leucine turnover, *Borsook, Deasy, Haagen-Smit, Keighley, and Lowy*, 1041  
 Protein, radioactive carbon-labeled alanine incorporation, effect, *Frantz, Zamecnik, Reese, and Stephenson*, 773
- Lymphoid tissue:** Antibody, release, *Roberts, Adams, and White*, 379
- Lysine:** L-, phosphatase, intestine, bone, and kidney, effect, *Bodansky*, 465  
 Synthesis, *Neurospora*, *Mitchell and Houlahan*, 883
- M**
- Magnesium:** Muscle adenosinetriphosphatase activation, relation, *Kielley and Meyerhof*, 387
- Malaria:** *See also* SN-3294, SN-13276
- Malate:** -Pyruvate oxidation-reduction, enzymatic mechanism, *Mehler, Kornberg, Grisolia, and Ochoa*, 961

- Malic acid: L-, decarboxylation, liver enzyme effect, Ochoa, Mehler, and Kornberg,** 979
- Manure: Chick growth factor, Bird, Rubin, and Groschke,** 611
- — —, stability and solubility, *Bird, Rubin, and Groschke,* 611
- Melanosarcoma: DL-Tyrosine labeled with radioactive carbon, distribution, Reid and Jones,** 427
- Meningococcus: Endotoxin, glucose phosphorylation, effect, Kun,** 761
- Methionine: -Deficient diet, DL-homoserine, growth, effect, Armstrong and Binkley,** 889
- Growth, leucine relation, *Harding and Shive,* 743
- Oxy-, lipotropic activity and toxicity, *Shaffer and Critchfield,* 489
- Methoxinine: Lipotropic activity and toxicity, Shaffer and Critchfield,** 489
- Methoxy - 1,4 - naphthoquinone: 2-, naturally occurring, isolation and antifungal action, Little, Sproston, and Foote,** 335
- Methyl alcohol: Blood acetol, administration effect, Grant,** 93
- Methyl group: Donor, dimethyl- $\beta$ -propiothetin, Maw and du Vigneaud,** 381
- , dimethylthetin, *du Vigneaud, Moyer, and Chandler,* 477
- Mold: See also Neurospora**
- Monomethyl-L-amino acid(s): De-methylation, oxidative, Ling and Tung,** 643
- Mosaic: Tobacco, virus, blood serum albumin and, interaction, Laufer,** 481
- Muscle: Adenosinetriphosphatase, magnesium-activated, Kielley and Meyerhof,** 387
- Myosin: Birefringence of flow, Binkley,** 385
- Myrothecium verrucaria: Enzyme, Saunders, Siu, and Genest,** 697
- N**
- Naphthoquinone: 2 - Methoxy - 1,4-, naturally occurring, isolation and Naphthoquinone—continued:**
- antifungal action, *Little, Sproston, and Foote,* 335
- Nephrectomy: Nitrogen metabolism study, use, Engel, Pentz, and Engel,** 99
- Neurospora: Growth, canavanine effect, Horowitz and Srb,** 371
- Lysine synthesis, *Mitchell and Houlahan,* 883
- Niacin: Tryptophan-, transformation, pyridoxine deficiency, effect, Ling, Hegsted, and Stare,** 803
- Tryptophan-deficient diets, amino acids, growth effect, *Hankes, Henderson, Brickson, and Elvehjem,* 873
- Nicotinic acid: Synthesis, chick embryo, tryptophan relation, Schweigert, German, and Garber,** 383
- Ninhydrin: Determination, colorimetric, enzyme kinetics, use in study, Schwert,** 411
- Nitrogen: Isotopic, tissue, adrenalectomy and fasting effect, White, Hoberman, and Szego,** 1049
- Metabolism, - nephrectomy effect, *Engel, Pentz, and Engel,* 99
- Nucleic acid: Desoxypentose, adenine incorporation, Brown, Petermann, and Furst,** 1043
- Desoxyribo-. See Desoxyribonucleic acid
- Pentose, adenine incorporation, *Brown, Petermann, and Furst,* 1043
- Synthesis, *Escherichia coli* B, bacteriophage-infected, *Cohen,* 281
- Yeast, cytidylic acid and diammonium uridylate preparation, *Loring, Roll, and Pierce,* 729
- Nucleotide: Triphosphopyridine, chemical constitution, Kornberg,** 1051
- Nucleotide pyrophosphatase: Chemical constitution, Kornberg,** 1051
- Nucleus: Cell, arginase, Dounce and Beyer,** 859
- Nutrition: Lactobacillus casei, factors, Daniel, Scott, Heuser, and Norris,** 71

## O

- Oxalosuccinic acid:** Preparation and properties, *Ochoa*, 115
- Oxalosuccinic carboxylase:** *Ochoa and Weisz-Tabori*, 123
- Oxidase:** Xanthine, related enzymes and, 6-pteridyl aldehyde effect, *Kalckar, Kjeldgaard, and Klenow*, 771
- Oxygen:** Dihydrodiphosphopyridine nucleotide and, electron transport, coupled phosphorylation, *Friedkin and Lehninger*, 757
- Oxymethionine:** Lipotropic activity and toxicity, *Shaffer and Critchfield*, 489

## P

- Palmitic acid:** Radioactive carbon-labeled, glucose from, alloxan diabetes, *Chaikoff, Lerner, Entenman, and Dauben*, 1045
- Pamaquine:** Determination, fluorometric, *Irvin and Irvin*, 589
- Dissociation exponents, *Irvin and Irvin*, 577
- Pantothenic acid:** *King and Cheldelin*, 273
- $\beta$ -Alanine utilization, relation, *King and Cheldelin*, 273
- Coenzyme A, determination, *Hegsted and Lipmann*, 89
- Propionic acid utilization, relation, *King and Cheldelin*, 273
- Penicillin:** Benzyl-, radioactive sulfur-containing, isolation, counter-current distribution use, *Sato, Barry, and Craig*, 217
- Crystalline, purity, counter-current distribution use, *Barry, Sato, and Craig*, 221
- Pentose nucleic acid:** Adenine incorporation, *Brown, Petermann, and Furst*, 1043
- Peptide:** Liver homogenate, isolation, *Borsook, Deasy, Haagen-Smit, Keighley, and Lowy*, 1041
- , leucine turnover, *Borsook, Deasy, Haagen-Smit, Keighley, and Lowy*, 1041
- Periodate:** Hydroxy-L-proline oxidation by, *Carter and Loo*, 723
- Phenol:** Amino groups, cross-linking, *Fraenkel-Conrat and Olcott*, 827
- Phenylalanine:** L-, decarboxylation, *Streptococcus faecalis* rôle, *McGilvery and Cohen*, 813
- Phosphatase:** Adenosinetri-, muscle, magnesium-activated, *Kielley and Meyerhof*, 387
- Bone, DL-alanine, L-glutamic acid, L-lysine, and L-histidine, effect, *Bodansky*, 465
- Intestine, DL-alanine, L-glutamic acid, L-lysine, and L-histidine, effect, *Bodansky*, 465
- Kidney, DL-alanine, L-glutamic acid, L-lysine, and L-histidine, effect, *Bodansky*, 465
- Pyro-, blood cell, red, *Naganna and Narayana menon*, 501
- , nucleotide, chemical constitution, *Kornberg*, 1051
- Phosphate(s):** Metabolism, shock effect, *Goranson, Hamilton, and Haist*, 1
- Phosphatidyl serine:** Chemical constitution, *Folch*, 439
- Phosphorus:** Bacteriophage desoxyribonucleic acids, origin, *Cohen*, 295
- Phosphorylation:** Coupled, dihydrodiphosphopyridine nucleotide and oxygen electron transport, *Friedkin and Lehninger*, 757
- Glucose, meningococcal endotoxin effect, *Kun*, 761
- Pituitary:** See also Hypophysectomy
- Potassium:** Isotopes, biological material, distribution, *Mullins and Zerahn*, 107
- Pregnancy:** Sulfonamide in diet, erythropoiesis in fetus, effect, *Williamson and Appel*, 769
- Pregnen-3 ( $\beta$ )-ol-20-one:** 5-, testis extract, isolation and identification, *Haines, Johnson, Goodwin, and Kuizenga*, 925

**Progesterone:** Blood serum solubility, *Bischoff and Pilhorn*, 663  
 Protein solution solubility, *Bischoff and Pilhorn*, 663  
 Salt solution solubility, *Bischoff and Pilhorn*, 663  
**Proline:** Derivatives, *Escherichia coli* mutant strains, utilization, *Simmonds and Fruton*, 705  
 Hydroxy-L-, oxidation, periodate, effect, *Carter and Loo*, 723  
**Propionic acid:** Utilization, pantothenic acid relation, *King and Cheldelin*, 273  
**Protein(s):** Blood serum, injury effect, *Gjessing, Ludewig, and Chanutin*, 683  
 -Formaldehyde reaction, *Fraenkel-Conrat and Olcott*, 827  
 Liver, radioactive carbon-labeled alanine incorporation, effect, *Frantz, Zamecnik, Reese, and Stephenson*, 773  
 Solutions, testosterone, progesterone, and  $\alpha$ -estradiol solubility, *Bischoff and Pilhorn*, 663  
 Synthesis, *Escherichia coli* B, bacteriophage-infected, *Cohen*, 281  
 —, — — — effect, *Melchior, Melody, and Klotz*, 81  
**Proteolysis:** Enzymes, tissue, *Gutmann and Fruton*, 851  
**Prothrombin:** Calcium effect, *Ware and Seegers*, 565  
 Purification, *Ware and Seegers*, 565  
 Thrombin effect, *Ware and Seegers*, 565  
 Thromboplastin effect, *Ware and Seegers*, 565  
**Pteridyl aldehyde:** 6-, xanthine oxidase and related enzymes, effect, *Kalckar, Kjeldgaard, and Klenow*, 771  
**Pteroylglutamic acid:** Analogues, *Woolley and Pringle*, 327  
 Pyrimidine derivatives, relation, *Hitchings, Elion, VanderWerff, and Falco*, 765  
**Pyridine:** Dihydrodiphospho-, nucleotide, oxygen and, electron trans-

**Pyridine—continued:**  
 port, coupled phosphorylation, *Friedkin and Lehninger*, 757  
**Pyridoxine:** Deficiency, tryptophanniacin transformation, effect, *Ling, Hegsted, and Stare*, 803  
 Displacement, *Martin, Avakian, and Moss*, 495  
**Pyrimidine:** Derivatives, pteroylglutamic acid, relation, *Hitchings, Elion, VanderWerff, and Falco*, 765  
**Pyrophosphatase:** Blood cell, red, *Naganna and Narayana menon*, 501  
 Nucleotide, chemical constitution, *Kornberg*, 1051  
**Pyrrolidonecarboxylic acid:** Polarimetry, *Kibrick*, 845  
**Pyruvate:** Isocitrate-, oxidation-reduction, enzymatic mechanism, *Mehler, Kornberg, Grisolia, and Ochoa*, 961  
 Malate-, oxidation-reduction, enzymatic mechanism, *Mehler, Kornberg, Grisolia, and Ochoa*, 961

Q

**Quinine:** Dissociation exponents, *Irvin and Irvin*, 577  
**Quinolylpiperidylcarbinol:** Dissociation exponents, *Irvin and Irvin*, 577

S

**Salicylate:** Blood, determination, fluorophotometric, *Saltzman*, 399  
**Salt:** Solutions, testosterone, progesterone, and  $\alpha$ -estradiol solubility, *Bischoff and Pilhorn*, 663  
**Sarcoma:** Melano-, DL-tyrosine labeled with radioactive carbon, distribution, *Reid and Jones*, 427  
**Scurvy:** Antipernicious anemia extracts and tyrosine metabolism, relation, *Sealock and Lepow*, 763  
**Serine:** Homo-. See Homoserine  
 Phosphatidyl, chemical constitution, *Folch*, 439  
**Shock:** Carbohydrate metabolism, effect, *Goranson, Hamilton, and Haist*, 1  
 Phosphate metabolism, effect, *Goranson, Hamilton, and Haist*, 1

- SN-3294:** Determination, fluorometric, *Irvin and Irvin*, 589
- SN-13276:** Determination, fluorometric, *Irvin and Irvin*, 589
- Sphingomyelin:** Blood serum, *Sinclair*, 343, 355
- Staphylococcus:** Toxin, solubility, *Wittler and Pillemmer*, 23
- Steroid(s):** Hormones, biological systems, *Bischoff and Pilhorn*, 663
- Stomach:** Mucosa, blood group A-specific substance, *Holzman and Niemann*, 305
- Streptococcus faecalis:** L-Phenylalanine decarboxylation, rôle, *McGilvery and Cohen*, 813
- Streptomycin:** Counter-current distribution, *Titus and Fried*, 57  
Tautomerism, *Titus and Fried*, 57
- Succinic:** Oxalo-, carboxylase, *Ochoa and Weisz-Tabori*, 123
- Succinic acid:** Oxalo-, preparation and properties, *Ochoa*, 115
- Sugar:** Blood. *See* Blood sugar
- Sulphydryl reagent(s):** Enzyme inhibition, mechanism, *Singer*, 11
- Sulfonamide:** Maternal diet, erythropoiesis in fetus, effect, *Williamson and Appel*, 769
- Sulfur:** -Containing amino acids, *Escherichia coli* mutant strains, utilization, *Simmonds*, 717  
Radioactive, benzylpenicillin containing, isolation, counter-current distribution use, *Sato, Barry, and Craig*, 217
- T**
- Testis:** Extract, *Haines, Johnson, Goodwin, and Kuizenga*, 925  
—, 5-pregnen-3( $\beta$ )-ol-20-one isolation and identification, *Haines, Johnson, Goodwin, and Kuizenga*, 925
- Testosterone:** Blood serum solubility, *Bischoff and Pilhorn*, 663  
Protein solution solubility, *Bischoff and Pilhorn*, 663  
Salt solution solubility, *Bischoff and Pilhorn*, 663
- Thetin:** Dimethyl-, methyl donor, *du Vigneaud, Moyer, and Chandler*, 477  
Dimethyl- $\beta$ -propio-, methyl donor, *Maw and du Vigneaud*, 381
- Thienylalanine:**  $\beta$ -2-, optical isomers, microbial growth, effect, *Ferger and du Vigneaud*, 241
- Thiobarbituric acid:** Lipide oxidation products and, reaction, *Bernheim, Bernheim, and Wilbur*, 257
- Thrombin:** Pro-. *See* Prothrombin  
Prothrombin, effect, *Ware and Seegers*, 565
- Thromboplastin:** Prothrombin, effect, *Ware and Seegers*, 565
- Thyroid:** Function, blood plasma inorganic iodide relation, *Wolff and Chaikoff*, 555
- Tobacco:** Mosaic virus, blood serum albumin and, interaction, *Lauffer*, 481
- Toxin(s):** Immunochemistry, *Wittler and Pillemmer*, 23  
*Staphylococcus*, solubility, *Wittler and Pillemmer*, 23
- Toxoid(s):** Immunochemistry, *Wittler and Pillemmer*, 23
- Tricarboxylic acid(s):** Biosynthesis, carbon dioxide fixation rôle, *Ochoa*, 115  
*Ochoa and Weisz-Tabori*, 123  
*Ochoa*, 133  
Carbon dioxide fixation, enzymatic mechanisms, *Ochoa*, 133
- Triphosphopyridine nucleotide:** Chemical constitution, *Kornberg*, 1051
- Trypsin:** Chymo-. *See* Chymotrypsin
- Tryptophan:** Chick embryo nicotinic acid synthesis, relation, *Schweigert, German, and Garber*, 383  
Metabolism, vitamin B<sub>6</sub> rôle, *Junqueira and Schweigert*, 605  
-Niacin transformation, pyridoxine deficiency, effect, *Ling, Hegsted, and Stare*, 803  
-Niacin-deficient diets, amino acids, effect, *Hankes, Henderson, Brickson, and Elvehjem*, 873
- Tyrosine:** D-, growth, effect, *Bubl and Butts*, 637

**Tyrosine—continued:**

- DL-, radioactive carbon labeled with,  
melanosarcoma, distribution, *Reid*  
and *Jones*, 427  
Metabolism, antipernicious anemia ex-  
tracts and, scurvy, relation, *Sealock*  
and *Lepow*, 783

V

- Van Slyke: Apparatus, blood gas, micro-  
volumetric, *Whiteley*, 947  
Vasoconstrictor: Blood serum, purifica-  
tion, *Rapport, Green, and Page*, 735  
Virus: Bacterial, synthesis, *Cohen*,  
281, 295  
Tobacco mosaic, blood serum albumin  
and, interaction, *Lauffer*, 481  
Vitamin(s): A, fetus, cholesterol-high  
maternal diet, effect, *Williamson*,  
631

**Vitamin(s)—continued:**

- A, utilization, media, *Halpern and*  
*Biely*, 817  
B<sub>1</sub>, tryptophan metabolism, rôle,  
*Junqueira and Schweigert*, 805  
B<sub>12</sub>, crystalline, chick growth, effect,  
*Ott, Rickes, and Wood*, 1047  
B<sub>12</sub>, distillers' solubles, growth, effect,  
*Novak and Hauge*, 647

X

- Xanthine: Oxidase, related enzymes and,  
6-pteridyl aldehyde effect, *Kalckar*,  
*Kjeldgaard, and Klenow*, 771

Y

- Yeast: Nucleic acid, cytidylic acid and  
diammonium uridylate preparation,  
*Loring, Roll, and Pierce*, 729



